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Immune Modulation of *Prevotella intermedia* Colonization in Squirrel Monkeys

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Colonization of the gingival crevice by black-pigmented *Porphyromonas* or *Prevotella* spp. (BP/P), including *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) and *Prevotella intermedia* (formerly *Bacteroides intermedius*), is thought to be an important ecological event which may result in the destruction of connective tissues supporting the teeth. Theoretically, periodontal diseases could be prevented if these or other periodontal pathogenic microorganisms did not colonize the subgingival area. The humoral immune response is one mechanism which may modulate bacterial colonization in the gingival crevice. In the present study, we tested the effect of systemic humoral immunity on subgingival colonization by indigenous *P. intermedia* in squirrel monkeys (*Saimiri sciureus*). Animals rendered essentially free of detectable BP/P by a single scaling, 10 days of tetracycline therapy, and toothbrushing three times per week were immunized with *P. intermedia* 1447 or were sham immunized with phosphate-buffered saline. Subsequently, all oral hygiene procedures were discontinued and five teeth in one quadrant were ligated with bacterium-soaked suture material to facilitate BP/P colonization. Immunization resulted in a significant increase in the level of immunoglobulin G anti-*P. intermedia* antibody in serum. Two weeks after ligation was initiated, *P. intermedia* could be detected in five of six sham-immunized and three of six immunized animals. Immunization was associated with a reduction in the emergence of indigenous *P. intermedia* in the gingival crevice.

Black-pigmented gram-negative anaerobic rods such as *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) and *Prevotella intermedia* (formerly *Bacteroides intermedius*), referred to as BP/P, are among the organisms that have been detected in subgingival plaque (3) and have been implicated as principal etiologic agents of periodontitis (26). Substantial microbiologic and immunologic data implicate *P. gingivalis* and *P. intermedia* in destructive forms of human adult periodontitis (16, 21, 26). Elimination or prevention of colonization by specific periodontal pathogens such as *P. gingivalis* or *P. intermedia* is thought to control the progression or onset of periodontal disease (22). Moreover, other bacterial diseases can be immunologically modulated by vaccination with whole bacteria or purified bacterial antigens (7). The presence of antibodies specific for periodontal microorganisms (25), as well as complement and polymorphonuclear leukocytes in the gingival crevice (2), suggests that components of the immune system required to immunologically modulate bacterial colonization can potentially come in contact with periodontal pathogens (18). Preliminary experiments suggest that it is possible to modulate colonization of periodontal pathogens in the gingival crevice. For example, Okuda and co-workers (17) have shown that the mean recovery of *P. gingivalis* (an organism not indigenous to rodents) in hamsters with ligated teeth can be significantly reduced by subcutaneous immunization with whole cells or by passive local immunization with rabbit anti-*P. gingivalis* or anti-bacterial hemagglutinin serum. Recently, using a nonhuman primate (*Saimiri sciureus*, the squirrel monkey) model, we demonstrated that immunization with *P. gingivalis* resulted in an increased level of immunoglobulin G (IgG) anti-*P. gingivalis* antibodies in serum and was associated with a significant reduction in BP/P colonization in the gingival crevice (13). We further observed that squirrel monkeys immunized with *P. gingivalis* harbored significantly fewer *P. gingivalis* organisms in the gingival crevice when compared with sham-immunized animals, but no difference in the levels of *P. intermedia* in the crevice was detected (13). The purpose of the present study was to assess the potential for vaccination with *P. intermedia* whole cells to modulate the recolonization or emergence of indigenous *P. intermedia* in immunized squirrel monkeys.

MATERIALS AND METHODS

Animals. Twelve adult female squirrel monkeys (*S. sciureus*), approximately 6 to 7 years of age, were used in these experiments. Animals were housed and fed as previously described (5) in the University of Florida's Health Center Animal Resources Department, in accordance with institutional guidelines. This facility is accredited by the American Association for Accreditation of Laboratory Animal Care. These animals had naturally occurring plaque and mild gingivitis and low levels of serum anti-*P. intermedia* IgG, IgM, and IgA (<20 U of each per ml). Before the immunization protocol was initiated, BP/P were detected in 10 of 12 animals or 30 of 48 quadrants. In an effort to reduce or eliminate detectable BP/P, animals were treated with dental prophylaxis and tetracycline therapy (20 mg/kg three times a day for 10 days). One week after the prophylaxis and antibiotic therapy had been completed, the animals were resampled to assess the effectiveness of therapy. A very low level (<1% of total cultivable microbiota) of BP/P was detected in only one quadrant of one animal (1 of 48 total quadrants). To maintain low or undetectable levels of colonization during the immunization regimen, the animals' teeth

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were brushed with 0.2% chlorohexidine gluconate three times per week.

Immunization. Monkeys (n = 6) were immunized as previously described for *P. gingivalis* 381 (13) but with 10^9 CFU of formalin-fixed (0.6% formalin) *P. intermedia* 1447. This strain had been derived earlier from the subgingival plaque of a squirrel monkey. The bacteria were emulsified in incomplete Freund adjuvant at a ratio of 0.5 ml of adjuvant and 0.5 ml of bacterial suspension. The teeth were ligated on day 0; therefore, the immunization protocol was begun 180 days before the teeth were ligated. We designated the day of the primary immunization as −180 days. One milliliter of *P. intermedia* 1447 (10^9)-incomplete Freund adjuvant was injected subcutaneously at four sites on the chest and abdomen of each animal. Sham-immunized animals received injections of 1 ml of emulsified incomplete Freund adjuvant without bacteria.

Immunized animals received three booster injections of 10^9 formalin-fixed *P. intermedia* at −145, −110, and −14 days before ligatures were placed in quadrant 4. Thus, immunization was completed before the ligatures were placed. These injections consisted of four subcutaneous injections on the chest and abdomen of 10^9 formalin-fixed *P. intermedia* cells suspended in phosphate-buffered saline solution (pH 7.2).

Specific antibody detection. Systemic IgG antibody responses to *P. intermedia* 1447 were monitored by a modified enzyme-linked immunosorbent assay (ELISA) (5, 8, 13, 19) in which the relative levels of specific antibody are expressed as units from a standard curve. Sera were collected at specific times and assayed for IgG anti-*P. intermedia* antibody by using *P. intermedia* 1447 sonic extract as the antigen on the plates. The data were reported as relative units of IgG anti-*P. intermedia* based on a standard curve of known amounts of monkey IgG bound to the test wells (4, 12). Specific antibody levels in saliva were not measured in this study.

Microbiology. Microbiologic sampling of the gingival crevicular area and culturing methods have been described previously (5, 13). Total cultivable microbiota and BP/P were enumerated on enriched tryptic soy agar (24). Immunoenzymatic differentiation was confirmed by biochemical tests and gas-liquid chromatography as previously described (5). Identification and differentiation of *P. gingivalis* and *P. intermedia* colonies were performed with immunoenzymatic detection by using species-specific monoclonal antibodies as described for *Actinobacillus actinomycetemcomitans* (14).

Ligation and inoculation. After the second booster injection (−110 days), toothbrushing procedures were stopped. During the 96-day interval between the second and third booster injections, the animals were sampled five times and remained essentially free of detectable BP/P; only 3 of 240 possible quadrants were minimally colonized (<1% BP/P). To facilitate recolonization or emergence of BP/P, the subgingival area around five teeth in the mandibular right quadrant (quadrant 4) of each animal was ligated with sterile 3-0 silk sutures 14 days after administration of the third booster injection. The day the ligatures were placed was designated day 0. At approximately 14-day intervals postligation, the animals were sampled for the presence of *P. intermedia* and *P. gingivalis*. In the nonligated quadrants (1 = maxillary right, 2 = maxillary left, 3 = mandibular left), sterile endodontic paper points were used to sample the gingival crevice around each tooth (three premolars and first and second molars). Samples from teeth in the same quadrant were pooled. In the ligated quadrant, ligatures around each tooth were removed and pooled together as the sample for that quadrant. After samples were taken, the ligatures were replaced in quadrant 4. The sampling and ligation regimen was repeated seven times over the next 100-day period.

TABLE 1. Anti-*P. intermedia* 1447 IgG antibody levels

Bleeding time (days postligation)	U of IgG/ml of serum[a] (mean [range])	
	Immunized	Sham immunized
0	5,813.2 (3,430–8,867)	30.7 (10–66)
35	4,043.7 (2,167–6,983)	104.8 (19–197)
70	3,181.0 (1,974–4,750)	145.7 (14–362)
91	2,479.5 (1,598–3,602)	111.3 (23–298)

[a] IgG anti-*P. intermedia* 1447 levels were determined with an ELISA, using *P. intermedia* 1447 sonic extract as the antigen.

Statistical methods. Although the *P. intermedia* and *P. gingivalis* responses are numerical in nature, they were clustered in three groups: zero, very low counts, or high counts. For analysis, we treated the data on a nominal scale as infected (low and high counts) or noninfected (zero counts). To evaluate differences in the frequency of occurrence of *P. intermedia* or *P. gingivalis*, we developed a repeated-measures regression model to compare the two groups, using all eight sampling times between day 0 and day 100. This method measures differences between monkeys by nesting within each group (13, 15). The analysis on this nominal response variable is appropriate since it produces an adequate sample size to compare sham-immunized with immunized monkeys across all time periods. All analyses were performed by the Statistical Analysis System (20).

RESULTS

Influence of immunization on levels of anti-*P. intermedia* 1447 in serum. Completion of the immunization protocol during the period −180 to −14 days prior to ligation resulted in levels of IgG anti-*P. intermedia* antibody in serum at 0, 35, 70, and 95 days after ligation which were clearly greater than respective levels in sham-immunized animals (Table 1). No additional injections were administered after the fourth injection at −14 days. The mean serum IgG anti-*P. intermedia* antibody response observed at ligation (0 days) was approximately 190-fold higher than that observed in the sham-immunized animals. Over the nearly 100 days during which teeth in quadrant 4 were ligated, the mean IgG anti-*P. intermedia* response declined by more than one-half the peak response in the immunized animals, while the mean level of IgG anti-*P. intermedia* antibody increased by more than threefold over the baseline levels in the sham-immunized animals (Table 1). However, the mean IgG anti-*P. intermedia* 1447 antibody levels in the immunized animals were always approximately 22-fold or greater than the mean IgG anti-*P. intermedia* 1447 antibody levels in the sham-immunized animals.

Influence of immunization on *P. intermedia* levels. Figure 1 reports the percentage of monkeys harboring *P. intermedia* at each time period by quadrant; as shown, *P. intermedia* was more frequently detected in sham-immunized than in immunized animals. Only at the first sampling after the ligatures were placed (day 16) were more immunized animals infected with *P. intermedia* (in one quadrant) than were sham-immunized animals. At that sampling, one immunized animal was infected with *P. intermedia* in quadrant 4 (ligated

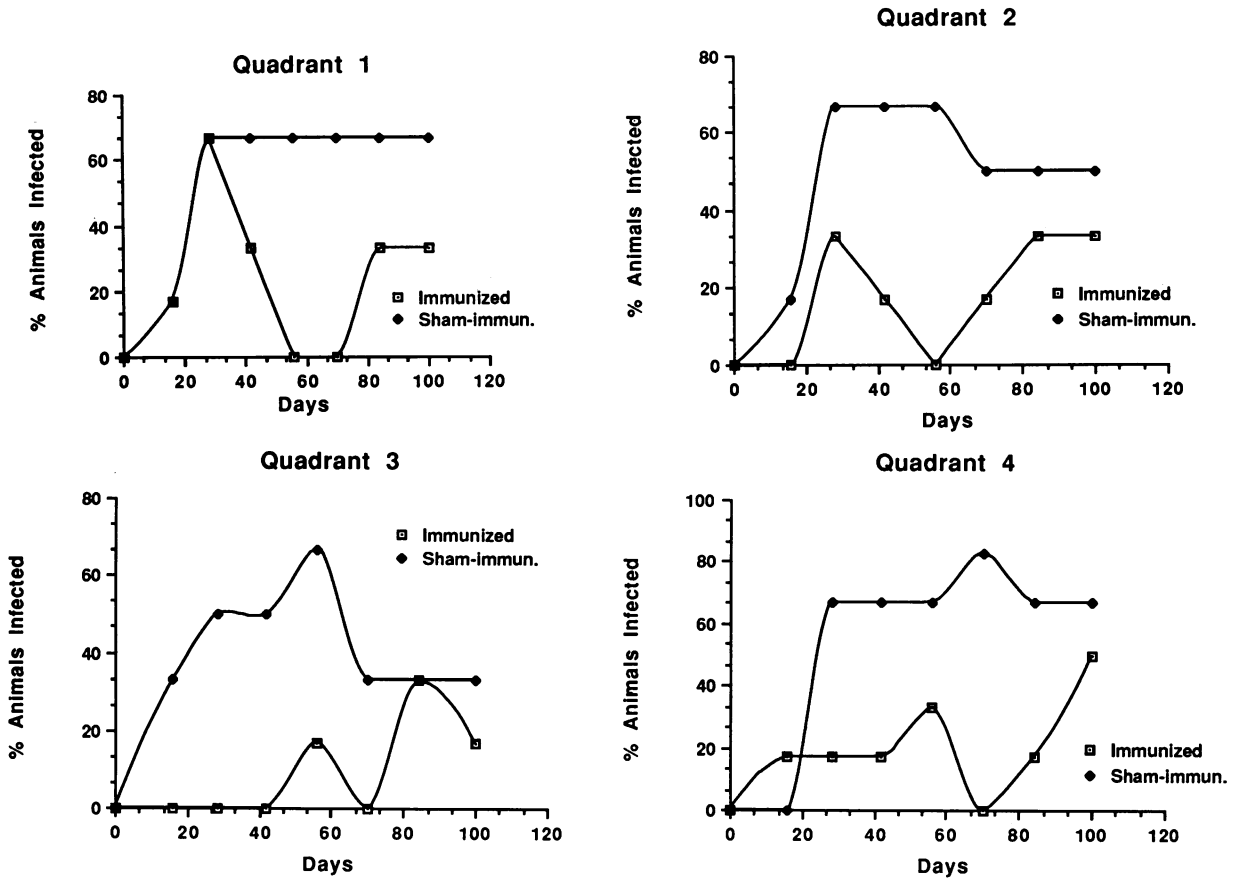

FIG. 1. Mean percentages of sham-immunized squirrel monkeys (n = 6) and squirrel monkeys immunized with *P. intermedia* 1447 (n = 6) harboring *P. intermedia* in subgingival plaque from quadrants 1, 2, 3, and 4 (ligated quadrant) at each sampling time.

quadrant) and no sham-immunized animals were infected in quadrant 4. At 56 and 70 days, the sham-immunized group exhibited a much greater frequency of *P. intermedia* colonization than did the immunized group in quadrants 1, 2, and 4. It is interesting that in the immunized group, *P. intermedia* tended to disappear by 56 and 70 days but then started to reappear, whereas in the sham-immunized animals, *P. intermedia* remained in high numbers throughout the study.

Data summarizing the number of quadrants colonized by *P. intermedia* and *P. gingivalis* for all dates in immunized and sham-immunized squirrel monkeys are shown in Table 2. The *P. intermedia* mean percentage of total microbiota in subgingival plaque for all dates is also shown for each quadrant (in parentheses, Table 2). The results of the repeated-measures analysis for each quadrant, in which differences among monkeys are measured by nesting within each group (13, 15), are presented in Table 2. Sham-immunized animals were more frequently colonized by *P. intermedia* than were immunized animals. As shown in Table 2, these differences were at or near statistical significance in all four quadrants. In contrast, there was essentially no difference between immunized and sham-immunized animals in the frequency of colonization by *P. gingivalis*.

DISCUSSION

These data demonstrate that immunization of squirrel monkeys with *P. intermedia* leads to an increased level of IgG anti-*P. intermedia* antibodies in serum and is associated with a reduction in the frequency of recolonization in the gingival crevice by indigenous *P. intermedia*. Before the immunization protocol was begun, animals were treated with mechanical and antimicrobial therapy to eliminate or reduce indigenous BP/P below the level of detection. After immunization was completed, the mandibular right quadrant (quadrant 4) was ligated with sterile silk suture material to

TABLE 2. Quadrants[a] colonized by *P. intermedia* in immunized and sham-immunized squirrel monkeys

Quadrant	No. colonized with *P. intermedia*/ no. of monkeys			No. colonized with *P. gingivalis*/ no. of monkeys	
	Immunized	Sham	P^b	Immunized	Sham
1	11/48 (0.7%)[c]	25/48 (1.6%)	<0.09	2/48	4/48
2	8/48 (0.8%)	22/48 (1.6%)	<0.07	2/48	1/48
3	4/48 (0.1%)	18/48 (0.3%)	<0.03	1/48	2/48
4	9/48 (1.5%)	25/48 (5.2%)	<0.08	3/48	2/48
Total	32/192	109/192	<0.06	8/192	9/192

[a] Data by quadrant are pooled for all weeks.
[b] Significance levels are from a repeated-measures regression model comparing groups after adjusting for monkey within-group effect.
[c] Number in parentheses is *P. intermedia* mean percentage of total microbiota in subgingival plaque pooled for all weeks by quadrant.

facilitate colonization or emergence of BP/P. *P. intermedia* could be detected in the animals within 16 days.

The cumulative results show differences in the number of infected sham-immunized and infected immunized animals that were at or near statistical significance in all quadrants (Table 2). Note that the number of animals (six immunized and six sham immunized) does not provide sufficient data for a statistical analysis for each time period. Also, of most interest is a comparison of the two groups over time: the six monkeys in each group over eight time periods produced 96 observations, enough for an adequate comparison of groups by quadrant. In addition, for each quadrant, the *P. intermedia* mean percentage of total microbiota in subgingival plaque was lower in immunized than in sham-immunized animals. Although differences between the immunized and sham-immunized groups in the number of animals infected seem similar in quadrants 1, 2, 3, and 4, the proportions of *P. intermedia* colonizing quadrant 4 are greater than in the other quadrants in both immunized and sham-immunized animals.

P. gingivalis colonization occurred infrequently in both groups, and there was no statistically significant difference between the groups. Since *P. gingivalis* infection in these animals was undetectable prior to initiation of this experiment (data not shown), it is not surprising that *P. gingivalis* infection remained low. In addition, *P. intermedia* and *P. gingivalis* do not cross-react, so we did not expect to detect a significant difference between immunized and sham-immunized squirrel monkeys in emergence or recolonization of *P. gingivalis*. However, these results are particularly interesting when considered with an earlier study which showed that colonization by *P. gingivalis* was significantly reduced in squirrel monkeys immunized and challenged with a monkey-derived isolate of *P. gingivalis* but that colonization of *P. intermedia* was not reduced (6, 13).

With the exception of quadrant 1, by day 28 fewer immunized than sham-immunized animals were infected with *P. intermedia*. Interestingly, after day 28, the number of infected sham-immunized animals remained fairly constant, whereas more immunized animals became infected after day 56. Since there are insufficient data for statistical analysis at each time period, we do not know whether the observation is statistically significant. However, assuming these observations are real, it is tempting to speculate that the decline in the amount of or bioactivity of serum anti-*P. intermedia* over time (Table 1) is responsible. Perhaps a booster injection during this time might have extended the period of suppression of *P. intermedia* in the immunized animals. Alternatively, the organisms colonizing after day 56 may differ antigenically or genetically from the original organisms (i.e., antigenic variation). However, neither of these possibilities can be confirmed in the present experiment.

The experimental design in the work reported here was quite different from that used in a previous study (13) from the standpoint of the mechanism of infection. The animals used in the present experiment harbored indigenous *P. intermedia*. Ligation was used to facilitate colonization or emergence of *P. intermedia* from an indigenous source. In the earlier experiments, infection was accomplished by weekly ligations and inoculations of the total dentogingival margin with viable *P. gingivalis*. Essentially nothing is known about the mechanisms of infection by the BP/P organisms in these animals. It could be argued that emergence of or reinfection by indigenous organisms may involve bacterial strains that are better adapted for infection (i.e., are possibly more virulent) than a selected laboratory strain isolated from a diseased site in a squirrel monkey, as was done in the previous study (13). Consequently, the modulation of an indigenous infection in conjunction with ligation may be more significant in light of the potential to limit natural infection in humans.

Ligature-induced periodontitis in the squirrel monkey (1, 9, 11), and other nonhuman primates (10, 23) has been well established. Although the microbial etiology of ligature-induced periodontitis in the squirrel monkey (or any of the other ligature models) has not been precisely established, BP/P have been implicated, and their colonization is strongly favored by the presence of ligatures in the gingival crevice (10, 23). We have also observed statistically significant increases in *P. gingivalis* and *P. intermedia* levels in ligated quadrants accompanied by significant alveolar bone loss relative to nonligated sites with minimal alveolar bone loss (6). Periodontal diseases are thought to be multifactorial diseases involving multiple etiologies (10, 16, 21–25). In theory, prevention of infection by one or more of the putative periodontal pathogenic organisms should prevent or reduce disease. The observation that in squirrel monkeys with ligated teeth whole-cell *P. intermedia* and *P. gingivalis* vaccines are associated with reductions in colonization or emergence of *P. intermedia* (in the present study) or *P. gingivalis* (in previous studies [13, 17]), demonstrates that it is possible to modulate infection by two prominent periodontal pathogens in a periodontal disease model. Obviously, the vaccination responses and influence on colonization need to be optimized. Theoretically, however, a multivalent vaccine against *P. intermedia* and *P. gingivalis* might reduce colonization by these organisms below a critical threshold necessary to initiate or maintain disease. Assuming that *P. intermedia* and *P. gingivalis* are directly involved in the initiation and/or progression of alveolar bone loss in this model, then immune modulation of the levels of these organisms in the subgingival crevice of squirrel monkeys below the critical threshold might also be expected to influence alveolar bone loss.

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