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Immunization against *Porphyromonas gingivalis* Inhibits Progression of Experimental Periodontitis in Nonhuman Primates

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Periodontitis is a common infectious disease in which the attachment tissues of the teeth and their alveolar bone housing are destroyed, resulting in tooth loss. The gram-negative anaerobic microorganism *Porphyromonas gingivalis* has been closely linked to severe forms of the disease. We show for the first time that immunization of the primate *Macaca fascicularis* with killed *P. gingivalis* in Syntex Adjuvant Formulation-M inhibits progression of periodontal tissue destruction.

Periodontitis is an infectious disease process that leads to destruction of the alveolar bone and connective tissues that support the teeth. The disease is highly prevalent in human populations worldwide, and it is a major cause of tooth loss (25, 26, 35). Treatment often involves frequent office visits to dental specialists over a long period of time. This is costly to both the patient and society, and new approaches for prevention and control which are more effective and which will reduce expense and suffering are badly needed.

Prominent among the bacteria associated with periodontitis is *Porphyromonas* (*Bacteroides*) *gingivalis* (20, 31, 42, 44). Many patients having severe periodontitis manifest serum antibodies reactive to antigens of their infecting bacteria (6, 14, 18, 29, 45, 47, 48). The role that these antibodies play in the progress of the disease is not known, although the bulk of the evidence indicates that they may be protective. Patients with high antibody titers have less severe disease and fewer affected teeth than those with low titers (11, 36). Serum antibodies in the presence of complement significantly enhance phagocytosis and the killing of periodontal pathogens by neutrophils in vitro (40, 46). Following periodontal treatment, previously seronegative patients seroconvert, and the capacity of their sera to stimulate phagocytosis and killing by neutrophils significantly increases along with increasing antibody titers and avidities (5, 32). These observations have led us to consider the possibility that immunization could have potential benefits in preventing and controlling periodontitis.

We report the results of a study with two objectives: can immunization with a vaccine containing killed *Porphyromonas gingivalis* suppress or eliminate *P. gingivalis* from the subgingival microflora, and of more importance, can immunization inhibit periodontal destruction as measured by loss of periodontal attachment and alveolar bone? Ligature-induced periodontitis in the cynomolgus monkey *Macaca fascicularis* was selected as the model in which to test the hypothesis. This model of periodontitis has been studied extensively in nonhuman primates, dogs, and rodents (9, 15, 16, 37, 38). Furthermore, several of the bacterial species associated with human periodontitis, including *P. gingivalis*, are present in the subgingival microflora of this animal, and destruction of the alveolar bone and connective tissue can be induced by oral implantation of *P. gingivalis* in *M. fascicularis* (13).

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MATERIALS AND METHODS

We screened 28 *M. fascicularis* monkeys residing in the Regional Primate Research Center at the University of Washington, Seattle, Wash., with regard to oral conditions, the presence and proportions of *P. gingivalis* in the subgingival flora, and serum antibody titers to antigens of *P. gingivalis*. Baseline data are reported in detail elsewhere (34). The protocol was approved by the Animal Care Committee, and animal care was done in accordance with the University of Washington Regional Primate Research Center guidelines. The overall design of the study protocol is shown in Fig. 1. We enrolled 20 monkeys, all of whom were colonized by *P. gingivalis*, had clinical manifestations of gingivitis but not periodontitis, and manifested low serum antibody titers to *P. gingivalis*. Ten were randomly assigned to an experimental (to be immunized) group (four females and six males), and ten were assigned to a control (placebo) group (four females and six males); 11 were wild trapped, and 9 were colony bred. The mean weight was 4.5 kg (range, 2.5 to 7.0 kg), and the mean age was 7.4 years (range, 4.6 to 11.0 years). Animals that are 4 to 5 years old are sexually mature. To relate the age of *M. fascicularis* to human age, one can multiply by a factor of 4 at the upper end of the age scale and by 5 at the lower end (46a). When in captivity, animals that are 11 years of age are not considered old. Student's *t* test of means showed that the control and experimental groups did not differ significantly with regard to plaque index, probing depth, percentage of *P. gingivalis* in the subgingival microflora, or specific serum antibody titer, but statistically the experimental group had significantly more gingival inflammation than the controls as assessed by the gingival index ($P < 0.001$). However, manifest gingivitis was found at 96% of all sites in the control group and at 100% of the sites studied in the experimental group (34).

The vaccine was prepared as follows. *P. gingivalis* 5083,

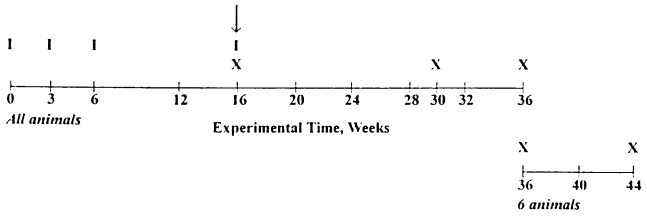

FIG. 1. Experimental plan. Twenty animals, 10 control and 10 to be immunized, were enrolled. Experimental animals were given complete vaccine; controls were given vaccine containing adjuvant but not *P. gingivalis*, at times indicated (I). Clinical evaluation was performed, and subgingival plaque samples and blood were harvested at each time noted. At week 36, a subset of three control and three experimental animals was superinfected with viable *P. gingivalis*, and the experiment was continued until week 44. Standardized radiographs for subtraction radiology were taken at the times indicated (X). Ligatures were placed in control and experimental animals at week 16 (indicated with an arrow).

isolated from *M. fascicularis* and provided by Stanley Holt, University of Texas, San Antonio, Tex., was grown anaerobically on blood agar plates for 7 days, harvested, washed three times in phosphate-buffered saline (PBS), and treated with 0.8% formaldehyde in PBS for 24 h at 4°C. The killed bacteria were harvested by centrifugation, and the formaldehyde was removed by washing; the bacteria were suspended in PBS at 0.5 mg of bacterial protein per ml and mixed with an equal volume of Syntex Adjuvant Formulation-M (SAF-M) provided by A. C. Allison and N. Byars, Syntex Laboratories, Palo Alto, Calif. (1, 2). Animals were injected at baseline and at 3, 6, and 16 weeks with 0.5 ml of vaccine intramuscularly in the upper arm and 0.5 ml subcutaneously in the back. Control animals were injected in the same manner with a vaccine containing adjuvant only.

At week 16, 000 braided silk ligatures (Ethicon; Johnson & Johnson, Somerville, N.J.) were placed subgingivally around the second premolar and first and second molar teeth in one mandibular and the contralateral maxillary sextant of each animal. At all times indicated in Fig. 1, clinical measurements were taken around test teeth, venous blood was collected, serum was prepared, and the subgingival microflora around ligated and nonligated test teeth was sampled by using paper points. We measured pocket depth and attachment level of test teeth, using the computer-assisted controlled force Florida probe (22), and plaque and gingival inflammation as described by Löe (19), except that the numerical range of the gingival index was extended to a maximum of four to account for very severe inflammation (30). At week 36, a subset of three control and three immunized animals was superinfected by applying a slurry containing approximately 10^7 viable *P. gingivalis* cells directly to the gingival margin area of ligated teeth, and the study continued through week 44.

Standardized periapical radiographs of mandibular test teeth were taken by one examiner throughout the course of the study using a parallel long-cone technique. Films were taken at week 16 when the ligatures were placed, at weeks 30 and 36, and for the subset of 6 animals at weeks 40 and 44. All films were processed in the same automatic film processor. Alveolar bone status was assessed by using computer-assisted densitometric image analysis (CADIA) (3, 21, 43). Standardized areas of marginal bone between the root surfaces of the mandibular first and second molars and between the first molar and second premolar were studied. The mean CADIA value for each sextant was used as the unit of observation. The CADIA method allows studies of positive and negative bone density changes within the defined area of interest by assessing changes in levels of grey. The CADIA method does not describe changes in marginal bone height. Therefore, the distance between the cementoenamel junction and the alveolar bone margin was measured on the mesial and distal aspects of the first molar and second premolar, by using the same films. The films were projected onto an opaque digitizing table at a linear magnification of $\times 10$. The alveolar crest was defined as the most-coronal bone level at which the periodontal ligament maintained its normal width (33). The distances measured were digitized for each image, and the bone height score was calculated in millimeters by using Sigma Scan 3.1 (Jandel Scientific, Corte Madera, Calif.). By using the animal as the unit of observation, mean values were calculated for ligated and nonligated sextants in immunized and nonimmunized animals.

To sample the subgingival microflora, one medium paper point (Johnson & Johnson) was placed to the bottom of the sulcus or pocket of each experimental tooth. The three paper points from each sextant were pooled into a single vial containing 1 ml of prereduced sterilized salt solution containing 0.04 M sodium chloride, 1.07 mM potassium chloride, 2.05 mM sodium thiosulfate, 0.47 mg of resazurin sodium salt per liter, and 4.12 mM L-cysteine-free base, to yield a single sample for each sextant. *P. gingivalis* DNA present in each plaque sample was measured by slot blot with a DNA probe provided by MicroProbe Corp. (Bothell, Wash.) as described in detail by Moncla et al. (27) and Dix et al. (8). Briefly, a series of standards consisting of total nucleic acids from *P. gingivalis* ATCC 33277 prepared as described by Moncla et al. (27) was used to normalize the test samples. The concentration of nucleic acids in the standards was determined spectrophotometrically (23) and then diluted and slotted to give nucleic acid concentrations ranging from 1 to 100 μg per slot. Hybridization with the DNA probe followed. The probe used had a sensitivity and specificity of 96 and 87%, respectively, for the monkey isolates of *P. gingivalis* (28). The data are presented as micrograms of *P. gingivalis* DNA per plaque sample.

To substantiate the presence of nucleic acid in each sample immobilized on a Nytran filter, we probed duplicate samples with a universal probe (MicroProbe Corp.). This probe binds to a conserved 16S rRNA region present within all bacteria (17, 27) and allows measurement of the total amount of nucleic acids immobilized on each slot of the Nytran filter, i.e., total bacterial load. By comparing the signal derived from the various diluted and immobilized standards of *P. gingivalis*, a direct estimate of the total amount of sample nucleic acid immobilized on the filter was obtained.

Serum immunoglobulin G antibody reactive with antigens of *P. gingivalis* was measured by enzyme-linked immunosorbent assay (ELISA) using *P. gingivalis* sonicate as the plate antigen, as described previously (39), and the titers were calculated by following the method of Butler (4).

RESULTS

Mean serum immunoglobulin G antibody titers for the 10 immunized and 10 control animals are shown in Fig. 2. The mean value for immunized animals was 12 ELISA units (EU) at baseline. Mean values increased to ~143 EU by the third injection at week 6 and then decreased by ~50% to a mean value of 67 EU by week 12. Following the fourth injection at week 16, the mean titer rose to 138 EU but decreased by more than 50% to 62 EU by week 36. The mean titer for control

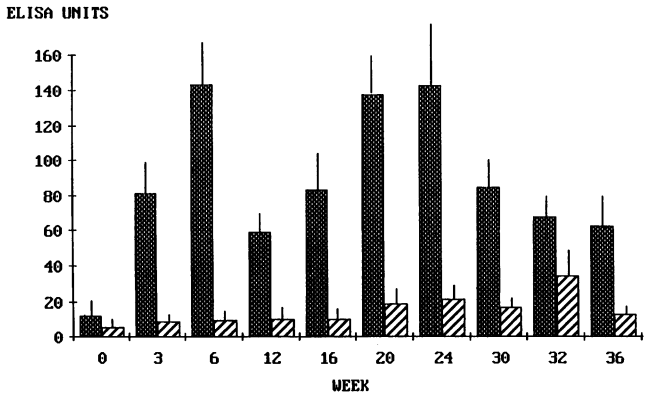

FIG. 2. Mean serum antibody titers reported as ELISA units (EU) and standard error bars for control and immunized animals at baseline and for weeks 3 through 36 following vaccination. ▩, immunized group; ▨, control group.

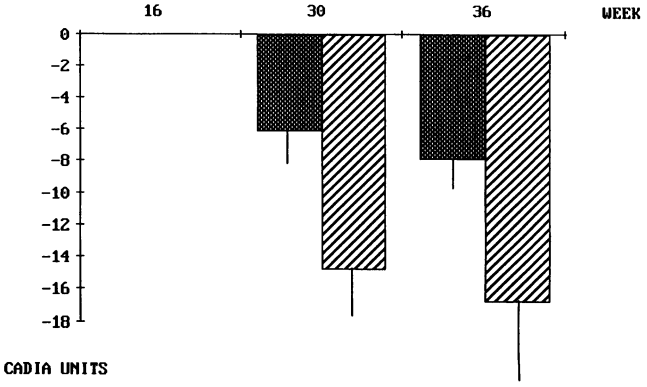

FIG. 4. Mean change and standard error bars in alveolar bone housing mandibular test teeth, reported in terms of CADIA units from week 16 when the ligatures were placed to week 36 (values at week 16 were considered as zero). ▩, immunized group; ▨, control group.

animals was 6 EU at baseline, and this value did not change significantly through week 36.

The mean amounts of *P. gingivalis* present in subgingival plaque of immunized and control animals in sextants to be ligated are shown in Fig. 3. Prior to ligature placement, vaccinated animals had slightly more *P. gingivalis* than control animals, but the difference was not significant. The amounts of *P. gingivalis* increased greatly following placement of ligatures at week 16 in both immunized and control animals. By week 30 and afterward, samples from ligated teeth in control animals had more *P. gingivalis* than samples from ligated teeth in immunized animals, but because of large intra- and interanimal variability and the relatively small number of animals used, the difference was not statistically significant when data for each time were subjected to the *t* test by using the animal as the unit of observation. No significant differences were observed in plaque scores or total bacterial DNA harvested from ligated teeth of control and immunized animals (data not shown). No significant differences were observed with regard to the mean micrograms of *P. gingivalis* DNA per sample for nonligated teeth in immunized and control animals (data not shown). Thus, immunization did not clear *P. gingivalis* infection from the subgingival areas, even at nonligated teeth.

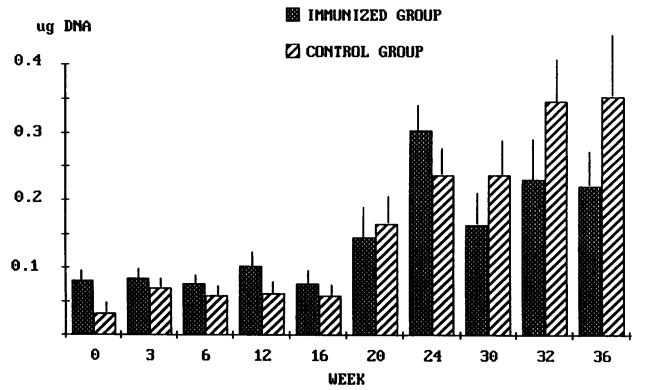

FIG. 3. Mean amount of *P. gingivalis* present and standard error bars for the subgingival microflora of immunized and control animals from ligated sextants at baseline and at 3 through 36 weeks, reported as micrograms of *P. gingivalis*-specific DNA per sample measured by slot blot with a *P. gingivalis*-specific DNA probe.

At week 36, the gingival inflammation scores were not significantly different for the immunized and control groups. Neither were determinations of attachment loss significantly different ($P < 0.07$), in part because of the small number of animals and lack of precision of the method used.

Bone area-density measurement results, which are the sums of degenerative and regenerative activities, are shown in Fig. 4 and reported as the assessed difference by CADIA between week 16 (zero level) and week 30 and 36 results. By weeks 30 and 36, the amount of alveolar bone loss was more than twice as great in control animals as that in immunized animals, and *t* tests demonstrated the difference to be significant ($P < 0.05$ and < 0.02, respectively). When mean bone heights for mandibular posterior sextants at week 16 were studied, no differences were found for ligated and nonligated teeth, between immunized and control animals or sextants. At week 36, the mean bone height for nonligated sextants did not differ between immunized and control groups. However, the mean difference in bone height indicating loss of bone between week 16 and week 36 was -0.4 mm (standard error, ± 0.1) for immunized animals and -0.8 mm (standard error, ± 0.1) for the control animals. This difference in bone loss was significantly different (*t* test, $P < 0.03$).

Data for the three control and three immunized animals which were superinfected by application of 10^7 viable *P. gingivalis* cells directly to the gingival margin area of ligated teeth are presented in Fig. 5. For the three immunized animals, the mean bone loss for mandibular teeth was approximately 4 CADIA units at week 36, and this value had not significantly changed at week 44. In marked contrast, the three control animals showed dramatic bone loss (14 CADIA units at week 36 and 22 CADIA units by week 44). Thus, the immunized animals appeared to be protected against a superinfection that induced progressive loss of bone in the control animals.

We observed a great deal of variability among our animals with regard to the kinetics and magnitude of the antibody response. This variation is illustrated in Fig. 6, by using data from representative animals. While the peak titer response for some animals, such as animal 1, was >400 EU, it was as low as 150 EU for other animals, such as animal 2. Some, such as animal 3, manifested their peak response at 20 weeks and failed to respond to any measurable extent to the injections at weeks 3 and 6; others, such as animal 2, responded to the first injection but manifested little or no response to the following injections until the injection at week 16. Still others, such as

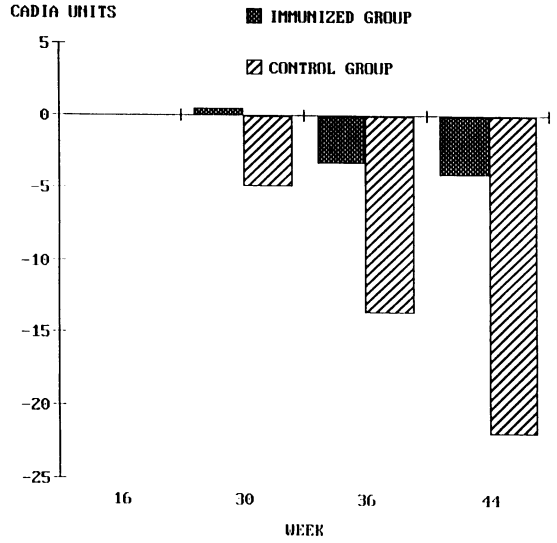

FIG. 5. Area-density values for alveolar bone housing mandibular test teeth, reported in terms of change in CADIA units from week 16, for control and immunized animals superinfected with *P. gingivalis* at week 36 and monitored at week 44.

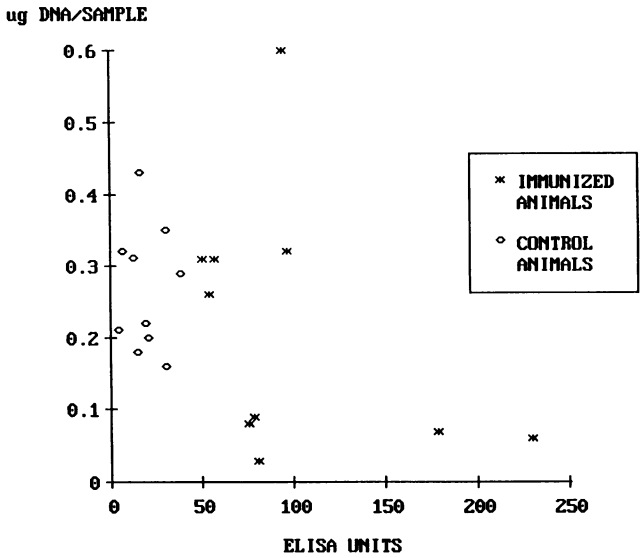

FIG. 7. Mean *P. gingivalis* values (in micrograms of DNA per sample) at week 36 plotted against mean serum antibody titers from the time of ligature placement at week 16 to the end of the experiment at week 36 for control and immunized animals.

animal 1, manifested peak titers at week 6 and again at week 20.

The observed variation in the magnitude and patterns of the titer response led us to calculate the mean titers from ligature placement at week 16 to the end of that experiment at week 36 and plot the values against the amount of *P. gingivalis* in the subgingival flora at week 36. As shown in Fig. 7, the vaccinated animals were segregated into two groups of five animals each: one with <0.1 μg of *P. gingivalis* DNA (group A), with a mean titer of 128 EU, and the other with >0.1 μg of *P. gingivalis* DNA (group B), with a mean titer of 68 EU. For group A, mean serum antibody titers were significantly greater ($P < 0.001$) and the numbers of *P. gingivalis* cells were significantly lower ($P < 0.001$) than those for control animals, while both values for group B did not differ significantly from those for controls.

DISCUSSION

Our study demonstrates for the first time that immunization can suppress periodontal tissue destruction in a primate model, as evidenced by measurement of alveolar bone loss, and provides evidence that development of a vaccine for use in prevention and control of periodontitis in humans may be possible. Our observations are supported by a report by Evans et al. (10), who demonstrated that immunization of gnotobiotic rats superinfected with *P. gingivalis* suppressed alveolar bone loss. Similarly, Chen et al. (6) were able to protect rats against *P. gingivalis*-induced skin abscess formation by vaccination. Our results differ greatly from those of Ebersole et al. (9), who assessed the effects of immunization of *M. fascicularis* using a vaccine containing killed *P. gingivalis* on the progress of periodontal tissue destruction. Although they were unable to document any statistically significant differences in the periodontal status of control and immunized animals, they concluded that immunization actually worsened the progress of the disease. Our study differed from theirs mostly in that we used the SAF-M adjuvant.

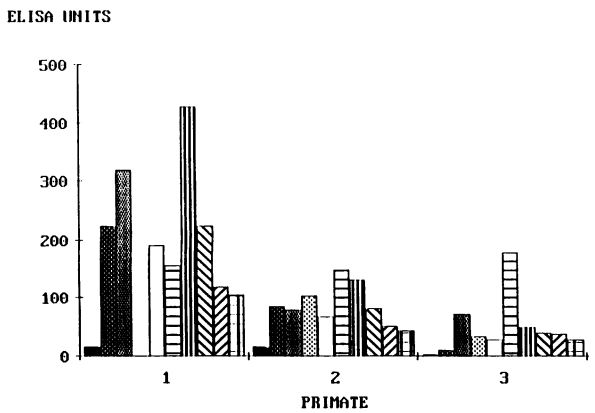

FIG. 6. Titers at each time for three representative immunized animals. Week 0 (■), 3 (▦), 6 (▩), 12 (⊡), 16 (□), 20 (▭), 24 (▭), 30 (▧), 32 (▨), and 36 (⊞).

Although vaccination elicited a relatively high mean antibody titer, high titers were not enduring. Mean titers for all immunized animals first peaked at week 6 when the third injection was given and then decreased by more than 50% by week 12. Mean titers peaked again at weeks 20 and 24 following the fourth injection at week 16 and then decreased by more than 50% by week 36. McArthur et al. (24) also noted a rapid decrease in serum antibody titers of squirrel monkeys immunized with *P. gingivalis*, although the antibody levels still were higher than those of controls. These observations are of concern in considering a vaccine for use in human periodontitis.

The magnitude and patterns of the antibody response were much more variable than indicated by the mean titer values. Some animals manifested a high peak titer of approximately 400 EU, while others manifested almost no response at all. Some animals manifested a peak response following the first two or three injections, others manifested a peak response following the fourth injection, and still others manifested two titer peaks. We cannot explain the wide variation in response except to say that while all animals had *P. gingivalis* in their

subgingival plaque prior to immunization, the amount varied greatly, and there was a statistically significant inverse correlation prior to immunization between specific anti-*P. gingivalis* antibody titers and the amounts of *P. gingivalis* (34). Interestingly, those animals that manifested the highest titers before vaccination also had the highest titers following vaccination. These observations indicate that as with human patients (6, 41, 48) and mice (12), some animals may innately be good immunologic responders capable of suppressing microbial overgrowth, while others may be poor immunologic responders who produce little antibody and who cannot suppress overgrowth.

We expected and observed a great deal of variability among our animals with regard to the extent of colonization by *P. gingivalis*. Although we observed considerably more *P. gingivalis* in the subgingival flora of ligated teeth of nonimmunized control animals than in immunized animals at weeks 32 and 36 (Fig. 3), these mean values were not statistically different because of the large between-animal variation and relatively small number of animals. However, the immunized animals segregated into group A, with <0.1 μg of *P. gingivalis* DNA, and group B, with >0.1 μg of *P. gingivalis* DNA. Serum antibody titers were significantly higher ($P < 0.001$) and *P. gingivalis* DNA values were significantly lower for ligated teeth in group A animals ($P < 0.001$) than control values, while values for group B animals did not differ from those for controls. These data indicate that some animals are innately good responders to *P. gingivalis* antigens while others are not and that good responders are able to suppress *P. gingivalis* overgrowth. Thus, while serum antibodies specific for *P. gingivalis* cannot clear *P. gingivalis* from the subgingival flora, when the titers are sufficiently high they do appear to suppress the *P. gingivalis* overgrowth around ligated teeth. Our observations are consistent with those reported by McArthur et al. (24) and Clark et al. (7), who attempted to block colonization of *P. gingivalis* and *Prevotella intermedia* in squirrel monkeys by immunization. They reported a trend toward less colonization in immunized animals, but they were unable to completely block colonization.

In spite of the extent of the interanimal variation and the fact that we used a minimal number of animals in each group, we were able to document a statistically significant reduction in alveolar bone loss both at week 30 and at week 36 in the immunized animals. We cannot say whether this reduction is a block or merely a suppression of bacterially induced bone destruction. We recorded a mean bone loss of about 6 CADIA units at week 30 in the immunized animals, and this had not increased significantly by week 36. This value is within the range that could be induced by the trauma of ligature placement alone, and it is also within the error of the method of measurement of bone area-density (3, 43). We believe that the data presented in Fig. 5 are of particular interest, even though only three control and three immunized animals were involved. Although superinfection with *P. gingivalis* resulted in rapid bone loss in the control animals, it had no measurable effect on CADIA scores for the three immunized animals. Thus, when all data are considered, immunization may in fact block bacterially induced bone loss. Additional studies with a larger number of animals are needed to confirm these findings.

The effects of immunization on gingival inflammation, probing depth, and attachment loss were not as clear-cut as the effects on bone. The presence of inflammation and subgingival ligatures in both immunized and control animals made clinical assessment of pocket depth and attachment levels difficult and can induce significant errors in assessments. We could not detect significant differences in mean inflammation scores or pocket depths and attachment loss as assessed by probing. Measurements of gingival inflammation and attachment level are considerably less precise than the method we used for detecting bone changes. Radiographic analysis, and specifically subtraction radiography, can be considered the standard against which periodontal disease activity can be measured. The data clearly demonstrate greater loss of bone in control animals, suggesting that immunization is protective.

The mechanism whereby immunization protects against alveolar bone destruction in ligature-induced periodontitis is unclear. We initially expected that immunization would suppress overgrowth of *P. gingivalis* and possibly lead to clearance of the organism from the flora. Some suppression was observed, but only in the high-titer animals; and even in these animals, very large numbers of *P. gingivalis* cells were still present at ligated sites. Thus, while serum antibodies specific for *P. gingivalis* may opsonize and enhance phagocytosis and the killing of *P. gingivalis* by neutrophils, additional mechanisms may also be involved. For example, bacterial components such as lipopolysaccharide may enter the tissues and activate macrophages to produce interleukin-1 and other mediators of bone destruction, as well as metalloproteinases that mediate destruction of the extracellular matrix, and immunization may block this activation. This hypothesis, while speculative, can be tested.

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