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Heterogeneity of *Porphyromonas gingivalis* strains in the induction of alveolar bone loss in mice

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These experiments examine alveolar bone loss in a model in which specific pathogen-free mice are exposed orally with *Porphyromonas gingivalis*. Alveolar bone loss was induced as a result of a specific infection with *P. gingivalis*, rather than other environmental antigens. Infection with live *P. gingivalis* was required, as significant bone loss did not follow gavage with formalin-killed *P. gingivalis*. The virulence of different strains of *P. gingivalis* was compared. Two laboratory strains of the bacteria (ATCC 53977 and W50) and a mutant strain lacking the 43-kDa fimbrillin (strain DPG3) induced bone loss. *P. gingivalis* 381, however, did not induce bone loss. There was a strong immunoglobulin G (IgG) antibody response to infection with each strain but a significant serum IgA response only to strain 381. These studies show that in mice with a background oral microflora bone loss is induced by a specific infection with *P. gingivalis* and that bacterial strain variation is important in determining whether alveolar bone loss will ensue.

Key words: bone loss; *Porphyromonas gingivalis*

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Periodontal diseases occur in humans and other animals and are infections accompanied by chronic inflammation (36). There is breakdown of the supporting structures of the teeth, including resorption of the alveolar bone of the jaw, resulting, ultimately, in loss of the teeth. Periodontal disease in adult humans is associated with the gram-negative, black-pigmented anaerobic bacterium *Porphyromonas gingivalis* (44, 49). Many potential virulence factors of *P. gingivalis* are known (4–6, 20, 24, 28, 31, 40, 42, 43, 49, 50). This bacterium has been demonstrated to affect many types of host cells, including immune cells (4, 5, 19, 35, 39, 41, 50) and cells involved in bone remodeling (17, 21, 26, 30, 37).

Periodontal disease progresses much more rapidly in some patients than in others, which may be due to host differences or to differences in virulence among different strains of *P. gingivalis* caused by genetic (27, 52), antigenic (23, 49) and/or functional (40) heterogeneity. Strains differ in their capacity to cause soft tissue destruction in animal models (7, 11, 15, 33, 47, 48).

Rodent gingival anatomy resembles that of humans (25); mice have been used as models for alveolar bone loss induced by bacteria other than *P. gingivalis* (10, 12, 13, 16, 29). *P. gingivalis* strains are known to differ in their ability to bring about alveolar bone loss in gnotobiotic rats (9), but heterogeneity of virulence has not been shown in mice with a background oral microflora. We have developed a specific pathogen-free mouse model in which oral delivery of *P. gingivalis* leads to reproducible alveolar bone loss (1–3). The present study has two aims. The first is to determine whether bone loss in this mouse model is a result of a specific infection with *P. gingivalis*. The second aim is to determine whether *P. gingivalis* strains differ in their ability to induce bone loss.

Material and methods

Animals

Specific pathogen-free BALB/cByJ mice were bred and raised at The Jackson Laboratory (Bar Harbor, ME). Animals were kept in the animal colony at Bates College under the accepted guidelines for animal care and maintenance and quarantined away from other animals. All mice were kept on a 12-h light/dark cycle and received distilled water and food ad libitum. Animals within an experiment were age-matched females, 8–12 weeks old at the start of experiments. All experiments were approved by the Animal Care and Use Committee, Bates College.

Bacteria

P. gingivalis ATCC 53977 (A7A1–28), 381 (2561; ATCC 33277), and W50 (ATCC 53978) were obtained from the culture collection of the Department of Oral Biology, State University of New York at Buffalo. Strain DPG3 was obtained from Todd Evans at SUNY Buffalo. That this bacterial strain lacks 43-kDa fimA fimbriae was confirmed by SDS-polyacrylamide gel electrophoresis (45). These strains are maintained at Bates College frozen in defibrinated sheep's blood at −70°C and by weekly transfer on supplemented blood agar (trypticase soy agar base with 0.1% yeast extract, 5.0 μg/ml hemin, 0.5 μg/ml menadione and 5% defibrinated sheep's blood). For experiments, bacteria were grown on supplemented blood agar at 37°C for 4–7 days in an anaerobic chamber (Forma Scientific, Marietta, OH) under an atmosphere of 5% CO_2, 10% H_2 and 85% N_2.

Oral infection

Mice were given sulfamethoxazole/trimethoprim (Sulfatrim®, Goldline Laboratories, Ft. Lauderdale, FL), 10 ml per pint in deionized water, ad libitum for 10 days. This was followed by a 3 day antibiotic-free period. Mice were then superinfected: 10^9 colony-forming units of live *P. gingivalis* in 100 μl of phosphate-buffered saline (PBS) with 2% carboxymethylcellulose were given by gavage (22) and direct inoculation of the oral cavity three times at 2-day intervals. Cage bedding was not changed during, nor for 10 days following, the gavage period. Controls included sham-infected mice which received the antibiotic pre-treatment and the carboxymethylcellulose, but no *P. gingivalis*. In one experiment, some mice were inoculated three times with 10^9 colony-forming units of *P. gingivalis* ATCC 53977 that had been killed by overnight room temperature incubation in 0.5% formalin and thoroughly rinsed in PBS prior to suspension in PBS and carboxymethylcellulose.

Infected mice were kept in a separate quarantine room apart from the sham-infected mice but under the same conditions of light and temperature. At the end of the experiment (47 days after the first gavage), mice were killed by CO_2.

Recovery of *P. gingivalis* from the murine oral cavity

At termination of the experiments, a sterile medium-sized paper point (Johnson and Johnson, East Windsor, NJ) was held against the gumline of the upper molars for 5 s. The paper point was placed in 1 ml of pre-reduced brain heart infusion broth supplemented with hemin and menadione and was vortexed gently for 10 s. An aliquot of 75 μl was plated onto supplemented blood agar and incubated anaerobically for 1 month. Plates were examined at 2 and 4 weeks. *P. gingivalis* colonies were identified by their black pigmentation and by Gram stain. While the colonies are usually visible by 2 weeks, the black pigmentation takes longer to develop (1).

Measurement of *P. gingivalis*–specific antibody

Blood was collected from each mouse at the time of euthanasia. Sera were stored at −70°C for later assessment of specific IgG, IgA and IgM antibody by enzyme-linked immunosorbent assay (ELISA). Ninety-six well polystyrene plates (Corning, Corning, NY) were coated overnight at 4 °C with the appropriate strain of *P. gingivalis* that had been killed in 0.5% formalin, extensively washed, then suspended in coating buffer to an optical density of 0.30 at 650 nm. Wells were washed in PBS with 0.5% Tween 20, incubated with two-fold serial dilutions (from $1{:}2^2$ to $1{:}2^{12}$) of test serum in PBS overnight at 10°C and washed to remove unadsorbed material. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG), IgA or IgM antibodies (Sigma Chemical Co., St. Louis, MO) were used to detect bound antibody. Bound alkaline phosphatase activity was quantified using *p*-nitrophenyl phosphate substrate and absorbance was determined at 405 nm by a microplate reader (Dynatech Model MR700). The ELISA titer was defined as the reciprocal of the highest serum dilution (expressed in $\log_2$) that produced absorbance readings more than 0.1 units above background levels. The mean ELISA titer for the sham-infected animals (generally zero for IgG or IgA and higher for IgM) was subtracted from the ELISA titer of each infected mouse to yield the specific antibody titer.

Measurement of alveolar bone loss

Horizontal bone loss around the maxillary molars was assessed by a morphometric method (1). Skulls were defleshed after 10 min treatment in boiling water under 15 lbs/inch^2 pressure, immersed overnight in 3% hydrogen peroxide, rinsed, air dried and stained with 1% methylene blue. The distance from the cementoenamel junction to the alveolar bone crest (CEJ:ABC) was measured at a total of 14 buccal sites per mouse. Measurements were made under a dissecting microscope (×40) fitted with a video image marker measurement system (model VIA 170, Boeckeler Instruments, Tucson, AZ) standardized to give measurements in mm. All bone measurements were done a total of three times in a random and blinded protocol by two evaluators.

The amount of bone lost was calculated as follows: the CEJ: ABC values from 14 sites were totalled for each mouse and subtracted from the mean total CEJ:ABC from sham-infected mice, to give the mm change in bone. Since the CEJ: ABC increases as bone is resorbed, this calculation gives negative values for mm change in bone when there has been bone loss.

Statistics

Differences between the infected and sham-infected groups in their CEJ to ABC distances and their antibody titers were evaluated by two-tailed, unpaired *t*-tests (Stat View: Abacus Concepts).

Results

Experiments were conducted to elucidate the capacity for *P. gingivalis* to induce alveolar bone loss in mice with a background oral flora. Comparisons were made to *P. gingivalis* ATCC 53977 (A7A1–28), originally isolated from a patient with insulin-dependent diabetes and adult periodontitis, which we have previously shown induces bone loss in specific pathogen-free mice (1). The strain is invasive in the abscess model (33) and induces bone loss in gnotobiotic rats (9).

In our mouse model the bedding in the mouse cages typically is not changed during the 5-day infection period nor for 7–10 days following the third gavage, to allow for possible recycling of the introduced bacteria by caprophagy. To determine whether

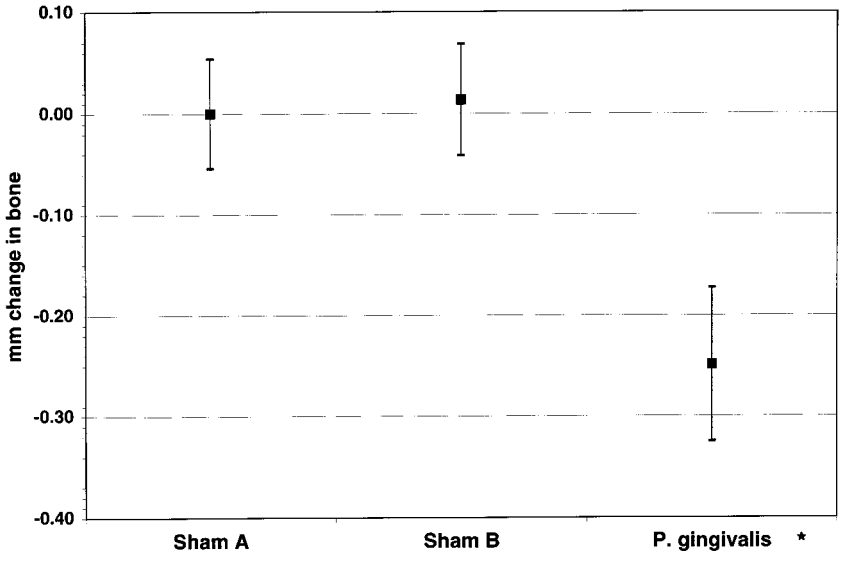

Fig. 1. Bone loss was a response to a specific infection with *P. gingivalis* ATCC 53977 and not a nonspecific response to environmental cage conditions. Negative values of mm change in bone indicate bone loss. *P. gingivalis*–infected mice and one group of sham-infected mice (Sham A) were kept in cages in which the bedding was not changed during the gavage period nor for 10 days following. Another group of sham-infected mice (Sham B) were kept in cages in which the bedding was changed more frequently, as was the bedding in all cages for the remainder of the 47 d experiment. Squares represent the mean of data from eight mice±SEM; ★=significantly different from Sham A at $P \leq 0.05$.

bone loss resulted from a specific infection with *P. gingivalis* ATCC 53977 rather than a response to environmental cage conditions, infected mice were compared to sham-infected mice kept in cages in which the bedding was either changed or not changed (Fig. 1). Sham-infected mice did not exhibit bone loss under either cage condition, while those mice gavaged with *P. gingivalis* did ($P<0.02$).

To determine whether induction of alveolar bone loss required live *P. gingivalis*, mice were gavaged with either live or formalin-killed *P. gingivalis* ATCC 53977. At the end of the experiment, mice that were orally infected with live bacteria had significant bone loss compared to either sham-infected mice or mice that received killed *P. gingivalis* (Fig. 2). Oral exposure to killed *P. gingivalis* induced a slight decrease in bone, but this did not reach significance ($P=0.17$).

Strains of *P. gingivalis* that have shown differences in virulence in other animal models were chosen for comparison to strain ATCC 53977. *P. gingivalis* strain 381 (genetically identical to strain 2561, ATCC 33277) (8) was originally isolated from an adult periodontitis patient (46). Despite being non-invasive or less invasive in mouse models for the soft tissue destruction which accompanies periodontal disease (11, 33, 48), *P. gingivalis* 381 induces bone resorption *in vitro* (21) and in gnotobiotic rats (9).

P. gingivalis fimbriae are important in bacterial adherence (14, 18, 23, 32), induction of cytokine secretion (32, 35), bone resorption (17, 21) and in infection and virulence (8, 28). *P. gingivalis* strain W50, a clinical isolate (38), was tested because its fimbriae are shorter than those on strain 2561 and are very few in number (45). W50 is highly invasive in the mouse abscess model (33, 48) yet does not induce bone loss in germ-free rats (R.T. Evans, personal communication). A mutant strain lacking the 43-kDa fimA fimbrial protein was also studied. This strain (*P. gingivalis* DPG3 derived from strain 381) does not cause bone loss in gnotobiotic rats (28).

We investigated the capacity of these strains of *P. gingivalis* to induce bone loss in specific pathogen-free mice with a background oral flora (Fig. 3). *P. gingivalis* ATCC 53977 induced bone loss, as did two other strains, *P. gingivalis* W50 and DPG3. One strain, *P. gingivalis* 381, did not induce bone loss.

Oral infection with each of the *P. gingivalis* strains produced a specific serum antibody response (Fig. 4). IgG was the dominant isotype after infection with most strains. Infection with *P. gingivalis* 381, however, produced a significant specific serum IgA response, equal in magnitude to the IgG response.

Differences in bone loss could not be

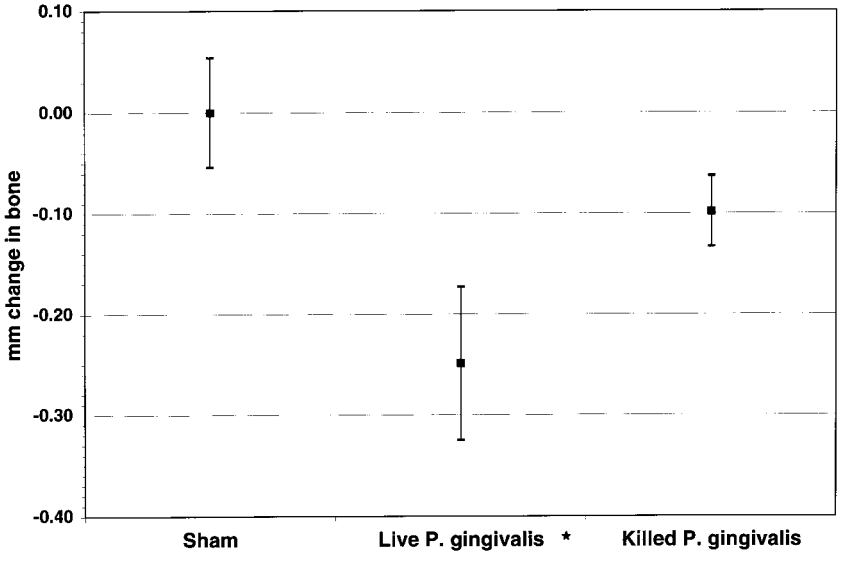

Fig. 2. Bone loss occurred in mice orally infected with live *P. gingivalis* ATCC 53977, but not in sham-infected mice nor in mice gavaged with formalin-killed *P. gingivalis* ATCC 53977. Negative values of mm change in bone indicate bone loss. Squares represent the means of data from eight mice±SEM. ★=significantly different from sham-infected mice ($P=0.018$). Data from mice exposed to killed *P. gingivalis* were not significantly different from data from sham-infected mice ($P=0.17$).

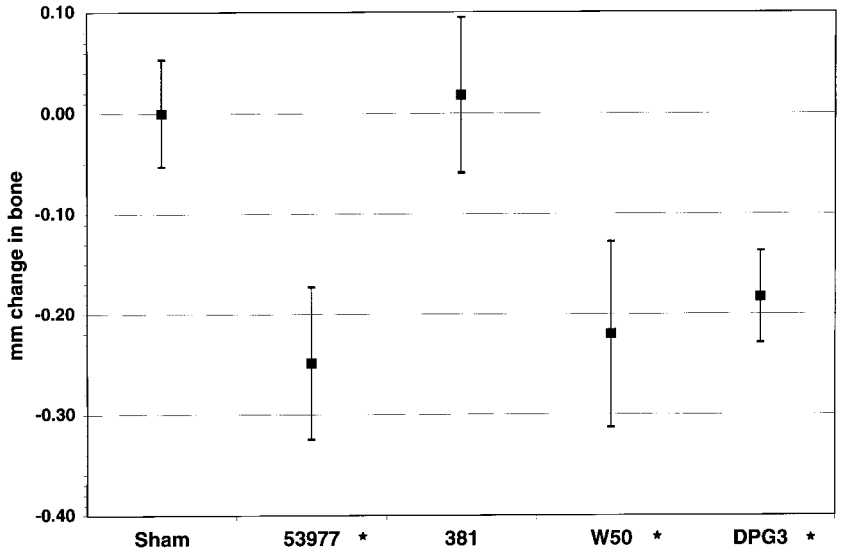

Fig. 3. Bone loss was induced by some, but not all, strains of *P. gingivalis*. Negative values of mm change in bone indicate bone loss. Squares represent the means from eight mice±SEM. ★=significantly different from sham-infected mice at $P<0.05$.

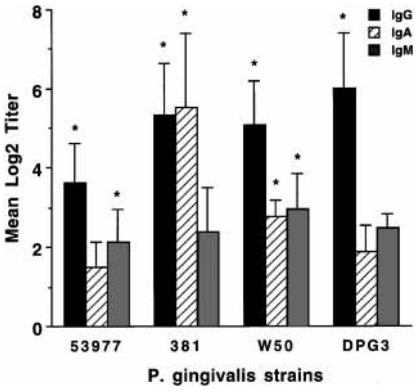

Fig. 4. Specific serum antibody responses to different strains of *P. gingivalis* in orally infected mice. Bars represent the means of data from eight mice±SEM. ★=significantly different from sham-infected mice at $P<0.05$.

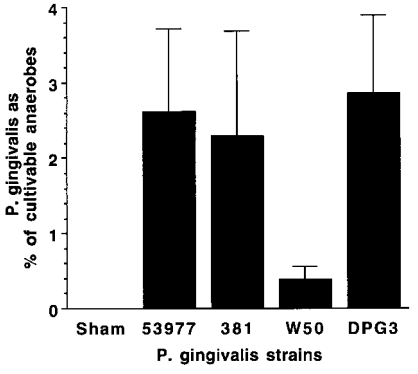

Fig. 5. Recovery of *P. gingivalis* from the oral cavities of mice at termination of experiment

explained by differences in infection load. *P. gingivalis* was recovered by paper point sampling from the infected mice at termination of the experiments (47 days post gavage). The amount of *P. gingivalis* is shown as a percent of the cultivable anaerobes in Fig. 5. *P. gingivalis* was not present in sham-infected mice. The percentage of *P. gingivalis* strain 381, which did not induce bone loss, did not differ significantly ($P>0.05$) from the percentage of the three strains that induced bone loss. The only group comparison that reached statistical significance was *P. gingivalis* strain W50 recovered in lower proportions than *P. gingivalis* strain DPG3 ($P=0.025$). Strains W50 and DPG3 induced comparable amounts of bone loss (Fig. 3).

Discussion

These studies show that oral infection with *P. gingivalis* is both necessary and sufficient for induction of alveolar bone loss in specific pathogen-free mice. Moreover, the studies show that bacterial strain variation is of considerable importance in determining whether or not alveolar bone loss will ensue. The only strain that failed to induce bone loss was *P. gingivalis* 381, in spite of the fact that it did colonize the oral cavity to the same extent as the other strains (Fig. 5).

It is conceivable that *P. gingivalis* 381 did induce bone loss in mice but not in a way detected by our morphometric technique, which measures horizontal bone loss. Intrabony interproximal defects, not detected by the morphometric method, can be measured by a radiographic method (22). *P. gingivalis* 381 induced more radiographically measured bone loss than did strain ATCC 53977 in gnotobiotic rats (9), but the radiographic technique is not sensitive enough to detect intraproximal defects in mice (personal communication, B. Klausen). In the rat studies, radiographic measurements had been made on the maxilla (22) and our measurements are typically made on the mandibular molars. Morphometric measurements on the maxillary molars (data not shown), however, revealed the same conclusions about bone loss in mice as did the mandibular measurements (Fig. 3). Moreover, the fact that strains W50 and DPG3 induced loss in specific pathogen-free mice but not in gnotobiotic rats argues against simple differences in measurement methods.

One of the most striking findings from this study was the fact that fimbriae do not appear necessary for induction of bone loss in specific pathogen-free mice, although they function in adherence (14, 18, 32) and in bone remodeling *in vitro* (21). Both a sparsely fimbriated *P. gingivalis* strain (W50) and a mutant that lacks the 43-kDa fimbrial protein (DPG3) induced bone loss, although they do not in gnotobiotic rats. These specific pathogen-free animals have a background oral flora. *P. gingivalis* coaggregates with several bacterial species (51), and coaggregation may play an important role in adherence in animals with a normal microflora, while it would not in germ-free animals. Despite its lack of fimbriae, DPG3 is normal for coaggregation and for another adherence factor, hemagglutinin (28). Non-fimbrillin factors may also stimulate bone loss. A 24-kDa fibroblast-activating factor from *P. gingivalis* W50 resorbs bone in *in vitro* assays (30), but its presence in other *P. gingivalis* strains has not been examined. The fact that *P. gingivalis* 381 failed to demonstrate bone loss while the mutant strain DPG3 that arose from 381 caused bone loss suggests that some undefined genetic alteration that distinguishes these two strains is critical for virulence.

Fimbriae from *P. gingivalis* 2561, genetically identical to strain 381, stimulate osteogenesis and bone resorp-

tion *in vitro* (21, 26), although strain 381 did not induce bone loss in mice (Fig. 3). The ability of a bacterial strain that did not induce bone loss *in vivo* to produce bone-remodelling *in vitro* may suggest that its virulence may be modified by the murine host response(s).

In this context, it is notable that serum IgA titers correlated with the pattern of virulence observed. Only the nonvirulent strain 381 caused a strong IgA antibody response, while all strains in this study induced a specific IgG response. IgA has anti-inflammatory properties, while IgG can be proinflammatory (34). IgG titers precede bone loss in this model but do not prevent it (2). It is thus possible that IgA, specific for fimbrillin or some other antigen, affords some degree of protection from bone loss. Immunization of gnotobiotic rats with fimbrillin raises high titers of antibody, which protects against bone loss (8); this antibody includes specific IgG and IgA.

In summary, we have shown that there are inherent differences in the capacity of strains of *P. gingivalis* to induce alveolar bone loss in specific pathogen-free mice after oral infection. Whether these differences in virulence indicate strain variation in bacterial structure or in the ability of the strains to induce protective immunity remains to be established.

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