Genetic Control of Susceptibility to *Porphyromonas gingivalis*-Induced Alveolar Bone Loss in Mice

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Periodontal disease affects a large percentage of the human population. Resorption of the alveolar bone of the jaw is a pivotal sequela of periodontal disease, because this bone is the attachment site for the periodontal ligaments that anchor the teeth. Using a murine model in which alveolar bone loss is induced by oral infection with *Porphyromonas gingivalis*, a gram-negative bacterium associated with human adult periodontal disease, we provide evidence suggesting that susceptibility to such bone loss is a genetically determined trait. AKR/J, DBA/2J, and BALB/cByJ or BALB/cJ mice were highly susceptible, while A/J, A/HeJ, 129/J, SJL/J, and C57BL/6J mice were much more resistant. When susceptible BALB/cJ and BALB/cByJ mice were crossed to resistant strains, two patterns were observed. (BALBc/ByJ × C57BL/6J)F$_1$ offspring were susceptible, suggesting C57BL/6J has recessive resistance alleles, while (BALB/cJ × A/J)F$_1$ mice were all resistant, suggesting that A/J mice have dominant resistance alleles. These results suggest a tractable genetic basis for *P. gingivalis*-induced alveolar bone loss and open the possibility of exploiting the mouse model to identify loci important for host susceptibility and resistance to periodontal disease.

Periodontal diseases are chronic inflammatory diseases which result in the breakdown of the supporting tissues of the teeth, including resorption of the alveolar bone of the jaw (32). Adult periodontitis is the most common form, but not all individuals are equally susceptible (7, 17). Attachment and bone levels decrease with age, but only 7 to 13% of individuals progress to advanced loss (15, 17, 21). Individuals living in similar environments develop disease at different rates, and 11% in a 15-year study showed no loss of attachment despite the presence of plaque and calculus and the lack of professional or self-care (22).

The gram-negative anaerobic bacterium *Porphyromonas* (*Bacteroides*) *gingivalis* is strongly associated with adult periodontitis (27, 30, 31). Again, not all individuals are equally susceptible to bone resorption when they are infected with this bacterium (17, 25), suggesting that host factors are important in the onset and progression of this disease.

Susceptibility to numerous infectious diseases appears to have a strong genetic component (23). Similarly, studies of adult-onset periodontal disease in humans suggest a genetic component (for reviews, see references 13 and 16). Studies involving monozygotic and dizygotic twins have suggested that about half of the population variance in disease could be accounted for by genetic factors (8, 24). Increased susceptibility to periodontal disease is found in conjunction with several inherited diseases (10, 28). Very recently, an interleukin-1 polymorphism has been reported to be a predictor of future severity in adult periodontal disease (19). With this possible exception, although many studies have pointed to genetic links with susceptibility to periodontal disease, no specific genes have been found (13).

Animal models, and murine models in particular, offer many advantages for analysis of genetic contributions to disease susceptibility. We have recently developed a murine model in which alveolar bone loss is induced after oral infection of specific-pathogen-free (SPF) mice with a human-derived strain of *P. gingivalis* (3, 4, 6). In this study, we have used this mouse model to determine whether inbred strains of mice and their F$_1$ offspring differ in their susceptibility to bone loss.

MATERIALS AND METHODS

Animals. SPF mice of strains A/J, A/HEJ, 129/J, SJL/J, C57BL/6J, AKR/J, DBA/2J, BALB/cByJ, and BALB/cJ were bred and raised at The Jackson Laboratory (Bar Harbor, Maine), as were F$_1$ mice from two crosses: CAF$_1$/J (F$_1$ of BALB/cJ females × A/J males) and CByB6F$_1$/J (F$_1$ of BALB/cByJ females × C57BL/6J males). The animals were kept in the animal colony at Bates College, Lewiston, Maine, under conditions described previously (3). The animals within an experiment were age-matched females, 12 weeks old at the start of experiments. The experimental protocol was reviewed and approved by the Animal Care and Use Committee, Bates College.

Bacteria for bone loss induction. *P. gingivalis* ATCC 53977 (A7A1-28) was maintained 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 of hemin per ml, 0.5 μg of menadione per ml, and 5% defibrinated sheep's blood). For experiments, bacteria were anaerobically grown under 5% CO$_2$, 10% H$_2$, and 85% N$_2$ on supplemented blood agar at 37°C for 4 to 7 days.

Oral infection. As described previously (3), mice were given sulfamethoxazole-trimethoprim (Sulfatrim; Goldline Laboratories, Ft. Lauderdale, Fla.) at 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 infected: 10^9 CFU of live *P. gingivalis* suspended in 100 μl of phosphate-buffered saline with 2% carboxymethylcellulose was placed in a feeding needle. Three times at 2-day intervals, half of this volume was placed down the throat of each mouse, and half was placed directly into its oral cavity. Controls included sham-infected mice which received the antibiotic pretreatment and the carboxymethylcellulose without *P. gingivalis*. Forty-seven days after the first gavage, mice were euthanized by CO$_2$.

Paper point sampling of the oral cavity. A sterile medium-sized paper point (Johnson and Johnson, East Windsor, N.J.) was held against the gumline of the upper molars for 5 s and then vortexed in 1 ml of prereduced brain heart infusion broth supplemented with hemin and menadione. A 75-μl aliquot plated onto supplemented blood agar was incubated aerobically or anaerobically for 4 weeks. *P. gingivalis* colonies were identified by their black pigmentation and by gram stain (3). Other species were distinguished from one another by colony morphology and gram stain.

Alveolar bone loss. Horizontal bone loss around the maxillary molars was assessed by a morphometric method (18). Horizontal bone loss is loss occurring in a horizontal plane, perpendicular to the alveolar bone crest, that results in a reduction of the crest height. Skulls were defleshed after 10 min of treatment in boiling water under a pressure of 15 lb/in^2, immersed overnight in 3% hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at a total of 14 buccal sites per mouse (Fig. 1). Measurements were made under a dissecting microscope (×40) fitted with a video image

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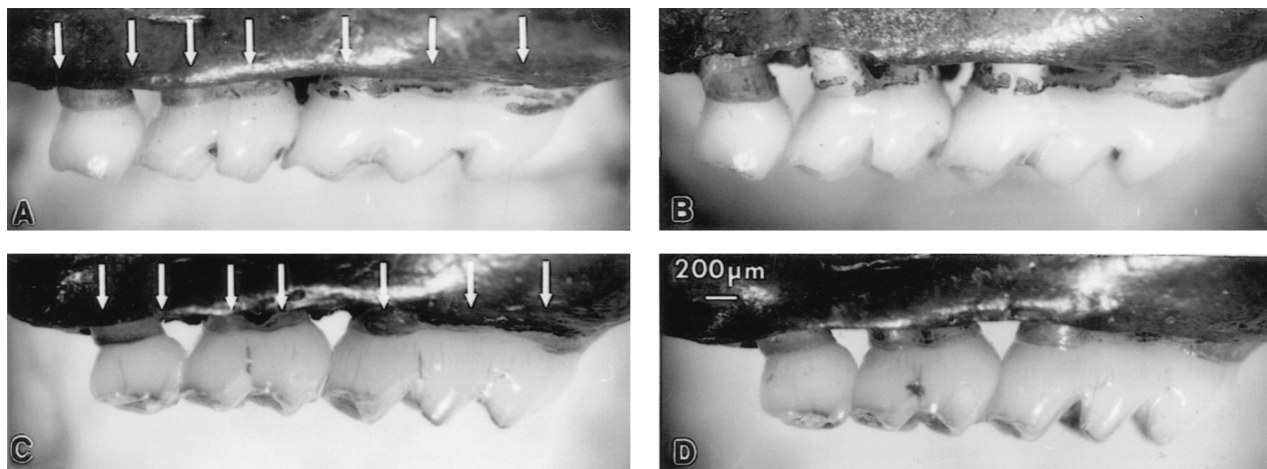
FIG. 1. Alveolar bone loss caused by oral infection with *P. gingivalis*. Measurement of bone levels is made comparing the distance from the CEJ to the ABC at seven buccal sites on the three molars on the left side and seven sites on the right side of the maxilla (shown by the arrows in panels A and C). (A) Sham-infected BALB/cByJ mouse. (B) *P. gingivalis*-infected BALB/cByJ mouse. As alveolar bone resorbs in infected mice, the distance from the CEJ to the ABC increases, becoming greater than in sham mice. (C) Sham-infected C57BL/6J mouse. (D) *P. gingivalis*-infected C57BL/6J mouse. When there is no bone loss, the distances from the CEJ to the ABC are the same in sham-infected and infected mice.

marker measurement system (model VIA 170; Boeckeler Instruments, Inc., Tucson, Ariz.) standardized to give measurements in millimeters. Bone measurements were done a total of three times in a random and blinded protocol by two evaluators.

The amount of change in the alveolar bone of each individual mouse was calculated by subtracting the CEJ-to-ABC distance (total of 14 sites) for that mouse from the mean CEJ-to-ABC distance of groups of sham-infected mice. Since the distance from the CEJ to the ABC increases as bone is resorbed, this calculation produces negative values for total millimeters of change in bone when bone loss has occurred.

Statistics. Differences between the infected and sham groups and between infected mice of pairs of strains were evaluated by two-tailed, unpaired *t* tests (Stat View; Abacus Concepts, Inc.).

RESULTS

To determine the effects of oral infection with *P. gingivalis* on alveolar bone, the distance from the CEJ to the ABC at 7 buccal sites per side (14 buccal sites per mouse) was measured after 47 days. Figure 1 shows one side of an upper jaw from a BALB/cByJ mouse orally infected with *P. gingivalis* (Fig. 1B) and from an age- and sex-matched mouse sham infected with carboxymethylcellulose rather than the bacterial preparation (Fig. 1A). Clear evidence of bone resorption was observed at several buccal sites, with the distance from the CEJ to the ABC greatly increased in the infected mouse compared to that in the sham-infected mouse. In contrast, infected C57BL/6J mice failed to show bone loss compared with their sham controls (Fig. 1D and C, respectively).

As can also be seen in Fig. 1, the alveolar anatomy differs among mouse strains (compare Fig. 1A and C). The CEJ-to-ABC distance in infected mice of different strains therefore cannot be compared directly, showing why it is important to compare levels in infected mice to those in sham-infected controls of the same strain. By subtracting the CEJ-to-ABC distance in an infected mouse from the mean CEJ-to-ABC distance in sham-infected mice, we can calculate the exact millimeter change in bone that can be attributed to the *P. gingivalis* infection.

Results comparing nine inbred mouse strains are summarized in Fig. 2. A/J, A/HeJ, 129/J, SJL/J, and C57BL/6J mice were more resistant to bone loss as a consequence of *P. gingivalis* infection than were AKR/J, DBA/2J, BALB/cByJ, or BALB/cJ mice.

However, it is important to note that in our experience, the amount of bone lost varies from one experiment to another, and resistant strains sometimes lose bone after infection, but they do so at fewer sites and in fewer mice than more susceptible strains (3). A/J appears to be more strongly resistant than C57BL/6J; in no experiment, has bone loss been initiated after *P. gingivalis* infection of A/J mice. When susceptible strains were run in the same experiment as resistant strains (as in the results shown below), the susceptible strains always lost more bone than the resistant strains after infection.

To determine whether resistance or susceptibility demonstrated Mendelian properties, F_1 mice from matings of susceptible strains with resistant strains were analyzed. The results are shown in Fig. 3, which shows the data from individual mice
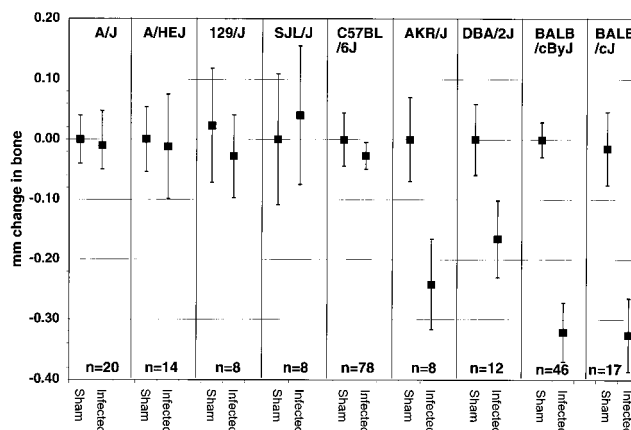

FIG. 2. Effect of *P. gingivalis* oral infection on alveolar bone levels in different strains of SPF mice. The values shown are the total millimeters of change at 14 sites (mean ± standard error). Negative values of millimeter change in bone indicate bone loss. Infected AKR/J, DBA2/J, BALB/cByJ, and BALB/cJ mice lost bone compared to sham controls ($P = 0.03$ for AKR/J, $P = 0.04$ for DBA/2J, $P = 2 \times 10^{-7}$ for BALB/cByJ, and $P = 0.006$ for BALB/cJ). In the other mouse strains, bone levels were not different in sham controls and infected mice ($P > 0.05$). *n*, number of mice tested. An equivalent number of *P. gingivalis*-infected and sham mice were tested per strain. When *n* is greater than 8, results are pooled data from 8 to 10 infected and control mice per experiment.
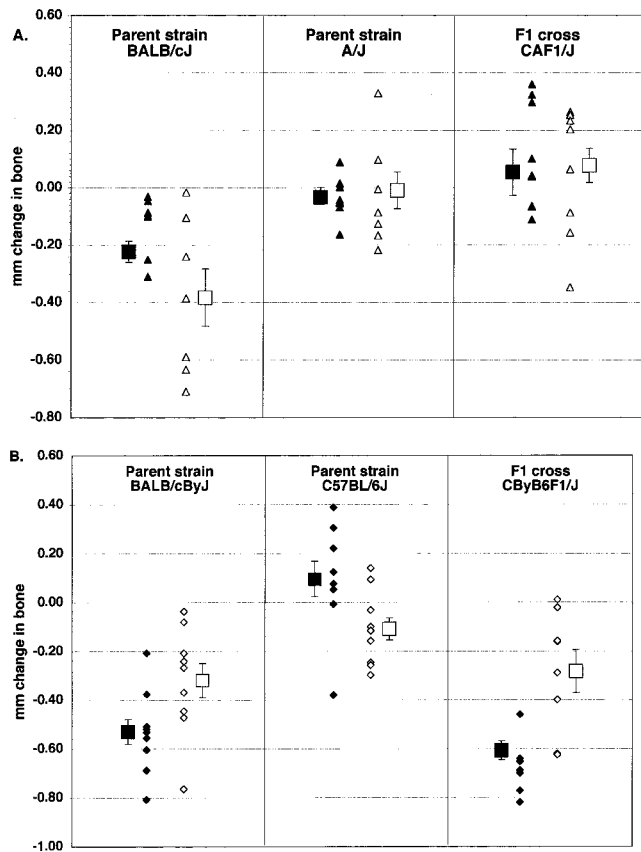
FIG. 3. Bone loss in *P. gingivalis*-infected F_1 mice from two different crosses. Each symbol represents one infected mouse. Data from sham-infected mice are not shown. Open symbols and solid symbols are results from independent experiments. Squares with brackets represent the mean ± standard error of the respective experiments. (A) BALB/cJ × A/J cross. Infected mice of one parent strain (BALB/cJ) lost bone (different from BALB/cJ sham controls at $P = 0.02$ in one experiment $P = 0.01$ in the other, and $P = 0.0006$ for pooled data); infected mice of the other parent strain (A/J) and infected F_1 mice (CAF$_1$/J) did not lose bone (not different from sham controls; $P > 0.05$). Values from infected BALB/c mice differed from those from infected A/J mice ($P = 0.031$ in one trial, $P = 0.028$ in the other, and $P = 0.0024$ for pooled data). Infected BALB/cJ mice also differed from infected CAF$_1$ mice ($P = 0.0049$ in one experiment, $P = 0.0041$ in the other, and $P = 6 \times 10^{-6}$ for pooled data). (B) BALB/cByJ × C57BL/6J cross. Infected mice of one parent strain (BALB/cByJ) and infected F_1 mice (CByB6F$_1$/J) lost bone. (Infected BALB/cByJ mice were different from BALB/cByJ sham controls at $P = 7 \times 10^{-6}$ in one experiment, $P = 0.01$ in the other, and $P = 9 \times 10^{-7}$ for pooled data; infected CByB6F$_1$ mice were different from CByB6F$_1$ sham controls at $P = 5 \times 10^{-7}$ in one trial, $P = 0.04$ in the other, and $P = 2 \times 10^{-5}$ for pooled data.) Infected mice of the other parent strain (C57BL/6J) mice did not lose bone (not different from sham controls, $P > 0.05$). Values from infected BALB/c mice differed from those from infected C57BL/6J mice ($P = 2 \times 10^{-7}$ in one trial, $P = 0.04$ in the other, and $P = 3 \times 10^{-7}$ for pooled data). Infected CByB6F$_1$/J mice also differed from infected C57BL/6J mice ($P = 3 \times 10^{-8}$ in one trial, $P = 0.05$ in the other, and $P = 3 \times 10^{-6}$ for pooled data).
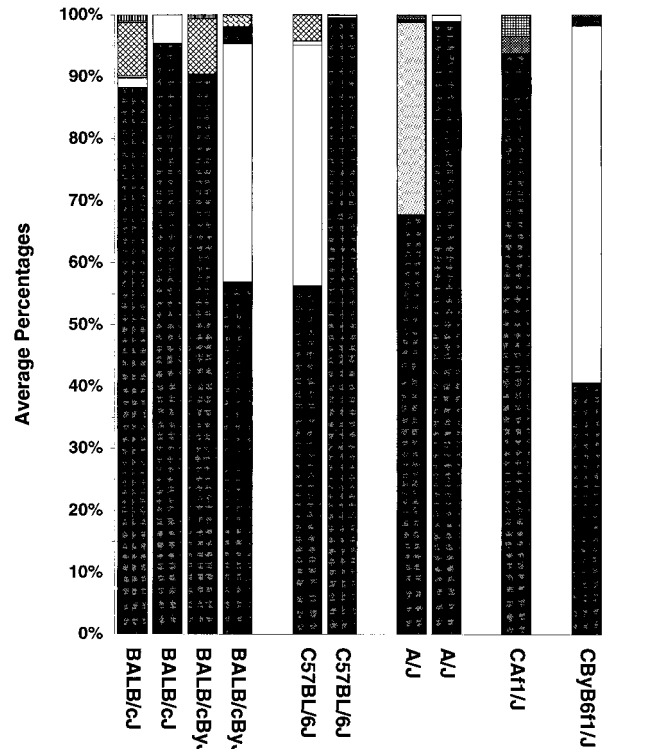

FIG. 4. Viable anaerobic microflora from various strains of mice immediately prior to infection with *P. gingivalis*. Duplicate bars for the same mouse strain are from two different experiments. Each pattern or tone of gray is a different bacterial species; the proportion of the bar shows the mean percentage of the total flora represented by that species.

as well as the calculated mean values. When BALB/cJ mice (susceptible) were mated with A/J mice (resistant), their F_1 offspring were highly resistant to bone loss after infection with *P. gingivalis* (Fig. 3A). In contrast, F_1 progeny of BALB/cByJ mice mated to the slightly less resistant strain (C57BL/6J) were susceptible (Fig. 3B).

Differences in bone loss could not be explained by differences in the background oral flora, either prior to superinfection with *P. gingivalis* or at the termination of the experiments. The data shown in Fig. 4 represent the oral anaerobic microflora as sampled by paper points immediately prior to superinfection with *P. gingivalis* (i.e., mice had been on antibiotics 10 days and off antibiotics 3 days). Based on colony morphology and gram stain, mice had a mixed oral flora of gram-positive and gram-negative species. *P. gingivalis* was never present prior to infection. One species predominated in every experiment except one, and this was a large gram-negative coccobacillus. There were generally low numbers of two to three other species present. There was more variation between experiments than there was among mouse strains. The mouse strains that were resistant to bone loss (C57BL/6J and A/J) had essentially the same background flora as did the bone loss-susceptible BALB/cJ and BALB/cByJ strains. Resistant CAF$_1$ mice had essentially the same flora as both the susceptible BALB/cJ and resistant A/J parent strains from the same experiment, as did susceptible CByB6F$_1$ mice and the susceptible BALB/cByJ and resistant C57BL/6J parent strains from the same experiment. Aerobic preinfection cultures revealed an additional two to three species of bacteria, all gram-positive cocci, which did not differ between mouse strains (data not shown). Aerobic or anaerobic cultures from paper point sampling done at the time of termination revealed a somewhat more complex flora, but again there were no differences among mouse strains (data not shown). The presence or percentage, either preinfection or at termination, of any bacterial species other than *P. gingivalis* did not correlate with bone loss in any mouse strain.

The presence of *P. gingivalis* at termination of the experiments correlated with bone loss in susceptible strains. The lack of bone loss in resistant strains could not, however, be attributed to smaller amounts of recoverable *P. gingivalis*. At termination, the percentage of *P. gingivalis* in the total recoverable
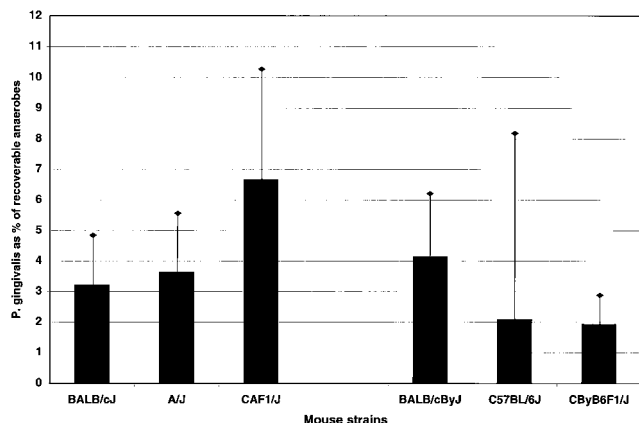
FIG. 5. Recovery of *P. gingivalis* from different mouse strains. *P. gingivalis* is shown as a percentage of the total anaerobic flora at the termination of the experiments. The first three mouse strains (BALB/cJ, A/J, and CAF$_1$/J) correspond to the strains in Fig. 3A, while the second three (BALB/cByJ, C57BL/6J, and CByB6F$_1$/J) are from the experiment shown in Fig. 3B. Bars represent the means from 10 mice ± standard error. No groups were significantly different ($P > 0.05$).

anaerobic flora (Fig. 5) and the number of *P. gingivalis* colonies per plate (data not shown) did not differ significantly between the susceptible (BALB/cJ) and resistant (A/J or C57BL/6J) mouse strains. The strain which tended to have the largest amounts of *P. gingivalis* was the resistant F$_1$ strain CAF$_1$/J, while the susceptible F$_1$ strain CByB6F$_1$/J tended to have the smallest amounts of *P. gingivalis*, although again, differences between strains were not statistically significant (Fig. 5).

DISCUSSION

Studies of the genetic basis of susceptibility and resistance to infectious disease are difficult to perform in humans. Mouse models are playing an increasingly prominent role in this endeavor. Common inbred strains often show differences in genetic predisposition to infectious diseases, and the mode of inheritance can be elucidated by genetic techniques regardless of whether the resistance or susceptibility trait is unigenic or polygenic (11, 23, 26).

Our results suggest that susceptibility to alveolar bone loss in mice as a consequence of exposure to *P. gingivalis* is a genetically determined trait. Bone loss was readily initiated in AKR/J, DBA/2J, BALB/cByJ, and BALB/cJ mice, while A/J, A/HeJ, 129/J, SJL/J, and C57BL/6J mice were more resistant. Early studies showed that mouse strains differ in their tendency toward spontaneous alveolar bone loss (1, 2). These studies differed from the present model in two important ways. Mice were not infected with *P. gingivalis*; uninfected mice of different strains were compared. Additionally, the mice were examined only at termination at 16 months. In the much shorter time course of our experiments, sham-infected mice showed no change in the CEJ-to-ABC distance (4). Sham-infected animals of different strains with different anatomy were not compared to each other, but were compared only to *P. gingivalis*-infected mice of the same strain. Thus, although there may also be genetic differences in bone breakdown over time, our model examined genetic influences on bone loss induced specifically by oral infection. Interestingly, mice of strain DBA/2A, which are resistant to spontaneous alveolar bone resorption (1, 2), are susceptible to *P. gingivalis*-induced bone resorption (Fig. 2).

Whether these results would only be induced by infection

with *P. gingivalis* is not known at this time. The presence of other bacterial species did not correlate with bone loss (Fig. 4); however, Gilbert and Sofaer have shown differences in mouse strains in alveolar bone loss after infection with another species of oral bacteria, *Actinomyces viscosus* (12). In their studies, bone levels in infected mice were compared to those in sham-infected mice of the same strain. Bone loss was maximal at a lower bacterial inoculum size than we used and was not induced by either higher or lower inocula. We have not seen this dependence on inoculum size (M. Dixon, unpublished observations). At the inoculum size which produced maximal bone loss in Gilbert and Sofaer's experiments, BALB/c mice lost more bone than did C57BL/6J mice, which is in agreement with our results.

Differences that we saw in bone loss among mouse strains are not likely to be due to differences in background flora (Fig. 4) or to *P. gingivalis* load (Fig. 5), because these did not show significant differences between resistant and susceptible mouse strains. We cannot entirely rule out these possibilities, however, due to the limits of the sensitivity of the paper point sampling method (N. Price, P. Baker, and U. Wikesjö, Int. Assoc. Dent. Res., abstr. 129, 1992).

In the experiments shown here, the results with the two F$_1$ crosses indicate that the genetic influences are likely to be complex. F$_1$ progeny were resistant or susceptible, depending on the cross. In a cross of BALB/cJ (susceptible) mice with A/J (resistant) mice, the F$_1$ mice were resistant, suggesting that resistance is dominant in this cross. However, when BALB/cByJ mice were mated with another resistant strain, C57BL/6J, the F$_1$ mice were susceptible, suggesting that resistance is recessive in this combination. Further crosses (F$_2$ and backcrosses) will be needed to establish any pattern of dominance. Quantitative trait locus analysis in conjunction with genome markers can be used to unravel genetic contributions whether they are unigenic or polygenic (9, 20). Our ability to measure bone loss as the millimeters of change in bone after infection makes this model amenable to quantitative trait locus analysis.

At this point, we do not know what specific loci may be involved. Susceptibility or resistance to many infectious diseases is dependent on genetically controlled differences in inflammatory responses, cytokine secretion, or T-cell recruitment after exposure to the pathogen (11, 26, 29). It is possible that genetically determined differences in immune regulation or in homeostatic bone remodeling are also important to the outcome of periodontal disease (14, 19). Our previous studies have shown that targeted knockout mice that are genetically deficient in CD4$^+$ T cells or in two T-cell cytokines (gamma interferon or interleukin-6) lose less bone in response to *P. gingivalis* infection than do immune-competent mice, while those that are deficient in the adhesion molecules ICAM-1 and P-selectin lose more bone (5, 6). Whether these are the traits that account for the observed differences between strains will become apparent during quantitative trait locus analysis.

Our findings of a heritable genetic basis for *P. gingivalis*-induced alveolar bone loss in mice provides a potentially important model to track the loci that confer susceptibility and resistance to alveolar bone loss that accompanies periodontal disease.

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