Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

J Clin Periodontol 2009; 36: 406–410 doi: 10.1111/j.1600-051X.2009.01393.x

Mouse model of experimental periodontitis induced by *Porphyromonas gingivalis*/*Fusobacterium nucleatum* infection: bone loss and host response

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Polak D, Wilensky A, Shapira L, Halabi A, Goldstein D, Weiss EI, Houri-Haddad Y. Mouse model of experimental periodontitis induced by Porphyromonas gingivalis/Fusobacterium nucleatum infection: bone loss and host response. J Clin Periodontol 2009; 36: 406–410. doi: 10.1111/j.1600-051X.2009.01393.x.

Abstract

Aim: To compare the effect of oral infection with *Porphyromonas gingivalis* or *Fusobacterium nucleatum versus* infection with both bacteria on mouse periodontal tissues, and to characterize the inflammatory response.

Materials and Methods: Mice were orally infected with *P. gingivalis*, *F. nucleatum* or both. At 42 days post-infection, alveolar bone loss was quantified using micro-computerized tomography. Tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β levels induced by the infection were quantified using the subcutaneous chamber model.

Results: Mice orally infected with *F. nucleatum*/*P. gingivalis* exhibited significantly more bone loss compared with that of mono-infected and sham-infected mice. *F. nucleatum*/*P. gingivalis* infection also increased the levels of TNF-α and IL1β compared with the levels found in the mono-infected groups.

Conclusions: Polymicrobial infection with *P. gingivalis*/*F. nucleatum* aggravates alveolar bone loss and induces a stronger inflammatory response compared with that observed upon infection with either bacterium alone. The results suggest that oral infection of mice with a mixture of *P. gingivalis* and *F. nucleatum* may be superior to mono-infection models of experimental periodontitis.

Key words: co-aggregation; *F. nucleatum*; inflammatory response; mixed infection; *P. gingivalis*

Accepted for publication 4 February 2009

Conflict of interest and sources of funding statement

The authors declare that they have no conflict of interests.
The present work is part of a PhD thesis by David Polak. The research was carried out at the MIS-supported Laboratory for Periodontal Research and The Ronald E. Goldstein Center for Esthetic Dentistry and Dental Materials Research, The Hebrew University-Hadassah Faculty of Dental Medicine, Jerusalem, Israel. The study was supported by a grant from the Chief Scientist of the Israel Ministry of Health.

Periodontal disease is characterized by an inflammatory process culminating in the destruction of the dental attachment apparatus. A growing body of evidence supports the notion that interactions, such as co-aggregation between different bacterial species, are important factors in periodontal disease pathogenesis (Holt & Bramanti 1991, Haffajee & Socransky 1994).

Porphyromonas gingivalis is a key microorganism in periodontitis, and its damage to the periodontium has been documented in many clinical and laboratory studies (Holt & Bramanti 1991, Socransky & Haffajee 1992, Okuda et al. 1994, Haffajee & Socransky 2005, Holt & Ebersole 2005). This bacterium has many virulence factors that enable it to manipulate the host-inflammatory response (Cutler et al. 1995). *Fusobacterium nucleatum* is also commonly cultivated from the subgingival plaque of inflamed gingivitis and from periodontal pockets (Haffajee & Socransky 1994), and participates in both adhesion and co-aggregation reactions with other microbial pathogens,

such as *P. gingivalis* (Bradshaw et al. 1998, Weiss et al. 2000).

Many investigations have been designed to study the virulence of several oral pathogens and their role in the aetiology of periodontal disease. Most of them involved mono-infection animal models (Kesavalu et al. 1992, Genco et al. 1998, Baker et al. 2000). Several studies have shown that co-infection with different pathogens modulates the immune response and affects the clinical outcome of the infection in various diseases (Vitovec et al. 2001, Stoicov et al. 2004). Using the mouse skin abscess model, Feuille et al. (1996) demonstrated a synergistic effect on soft tissue destruction by mixed infection with *P. gingivalis* and *F. nucleatum*. Destruction of the soft tissue continued long after the bacterial infection with *P. gingivalis* and *F. nucleatum* was eradicated (Ebersole et al. 1997). More recently, a study on rats, using the oral infection model, has shown that oral infection with *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *F. nucleatum* together induced robust alveolar bone loss compared with that obtained upon infection by either bacterium alone (Kesavalu et al. 2007). The bone loss did not differ when *F. nucleatum* was excluded from the infection.

We hypothesize that the polymicrobial infection enhances virulence, both accelerating and aggravating bone loss, compared with that occurring upon infection with one bacterium. Our objectives were to determine the effect of oral infection with *P. gingivalis*, *F. nucleatum* or both on the mouse periodontal tissues, at the molecular and clinical levels.

Materials and Methods

All experiments were performed in the SPF unit of The Hebrew University-Hadassah Medical Center, and approved by the university's Animal Care and Use Committee.

Bacterial cultivation. *P. gingivalis* strain ATCC 33277 and *F. nucleatum* strain PK 1594 were grown in Wilkins broth (Chalgren broth, Oxoid Ltd, Cambridge, UK), in an anaerobic chamber with 85% N_2, 5% H_2 and 10% CO_2, followed by three washes in phosphate-buffered saline (PBS). The bacterial concentration was measured spectrophotometrically (Kolenbrander & Andersen 1989, Genco et al. 1991).

Oral infection (Baker et al. 1994). Four- to five-week-old Balb/C mice were superinfected three times at 2-day intervals with *P. gingivalis* ($\sim 400\,\mu$l of a 10^9 bacteria/ml suspension for each mouse), *F. nucleatum* ($\sim 400\,\mu$l of a 10^9 bacteria/ml suspension for each mouse) or a mixture of both ($\sim 400\,\mu$l of a 10^9 bacteria/ml suspension made up of equal volumes of each bacterium), and 42 days after the last gavage the maxillary jaws were harvested.

Micro-computerized tomography (CT) analysis. Maxillary hemi-jaws were analysed by compact fan-beam-type CT (μCT 40, Scanco Medical, Bassersdorf, Switzerland) as described previously (Wilensky et al. 2005).

The subcutaneous chamber model was used to evaluate the local inflammatory response to bacterial challenge as described previously (Houri-Haddad et al. 2000). Four- to five-week-old Balb/C mice were challenged by an injection of $100\,\mu$l of PBS containing *P. gingivalis* (10^6 bacteria) or *F. nucleatum* (10^6 bacteria) or both (a total of 10^6 bacteria containing 5×10^5 of each bacterium). Chamber exudates were harvested at baseline (before bacterial injection), and 2 and 24 h post-challenge for cytokine analysis.

Enzyme-linked immunosorbant assay (ELISA) of cytokines. The secreted forms of mouse tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β were quantified using two-site-ELISA, the method based on commercially available antibody pairs (Pharmingen, San Diego, CA, USA), as described previously (Houri-Haddad et al. 2000). The range of detection for each specific cytokine was 25–2000 pg/ml.

Co-aggregation assay. A suspension of *F. nucleatum* and *P. gingivalis* (50μl each) was mixed vigorously for 10 s and a visual rating scale of 0–4 was used to grade the reaction. A score of 0 signified an evenly turbid suspension with no visible aggregates, i.e., no co-aggregation, and a score of 4 signified maximal clumping, leaving a clear supernatant (Weiss et al. 2000).

Data analysis. The animal studies were carried out with at least six mice per treatment group. The data were analysed using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated measure analysis of variance (RM ANOVA) was used to test the significance of the differences between the treated groups. If the results were significant, inter-group differences were tested for significance using Student's *t*-test and the Bonferroni correction for multiple testing.

Results

P. gingivalis and *F. nucleatum* co-aggregate in vitro and in vivo

The synergistic effect between two bacteria may depend on their ability to co-aggregate in vivo. First we examined the ability of *P. gingivalis* and *F. nucleatum* to co-aggregate in vitro. The level of co-aggregation in vitro between *P. gingivalis* and *F. nucleatum* was 2–3 on a scale of 0–4. To ascertain that the two bacteria can also co-aggregate in vivo, a mixture of both bacteria (10^8 bacteria) was injected into subcutaneous chambers in mice (Houri-Haddad et al. 2000). The chambers were sampled 2 and 24 h post-infection, and examined under phase microscopy ($\times$ 1000). Co-aggregation of *P. gingivalis* and *F. nucleatum* was evident at the two tested time intervals (Fig. 1).

P. gingivalis/*F. nucleatum* infection induces alveolar bone loss

We tested the ability of the two bacteria to induce experimental periodontitis in mice. Mice were orally infected with *P. gingivalis*, *F. nucleatum* or both, while control mice received vehicle (PBS) only. Under the present experimental conditions, mice infected with *P. gingivalis* or *F. nucleatum* alone did not exhibit significant alveolar bone loss, which was essentially similar to that in the non-infected animals (Fig. 2). However, mixed infection with *P. gingivalis*/*F. nucleatum* induced significant alveolar bone loss, compared with that found in the mono-infected groups and the sham-infected control group. The residual bone volume (as measured by volumetric micro-CT analysis) in the mixed-infection group was $\sim 70\%$ of the bone level found in the other three groups (Fig. 2).

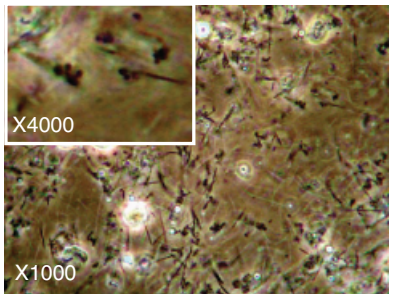

Fig. 1. Light microscopy of chamber exudates following mixed infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Mice received an intra-chamber challenge of a mixture of *P. gingivalis* (10^8 bacteria) and *F. nucleatum* (10^8 bacteria). Chamber exudates were harvested at 24 h post-infection and examined under phase microscopy at × 1000 and × 4000 magnification.

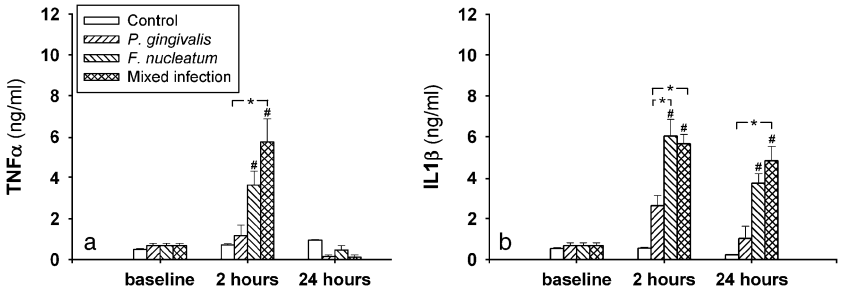

Fig. 3. Inflammatory cytokine response. Mice ($n = 6$ in each group) received an intra-chamber challenge of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* or a mixture of the two. The control group was challenged with saline alone. Chamber exudates were obtained at baseline (before challenge), and at 2 and 24 h post-infection. The levels of each cytokine in the chamber exudates were determined by an enzyme-linked immunosorbant assay. The results are expressed as the mean ± standard error. #, a significant difference from the control group; *, a significant difference from the other groups. $p < 0.05$. (a) Tumour necrosis factor-α levels induced by mono *versus* mixed infection. (b) Interleukin-1β levels induced by sub-clinical mono *versus* mixed infection.

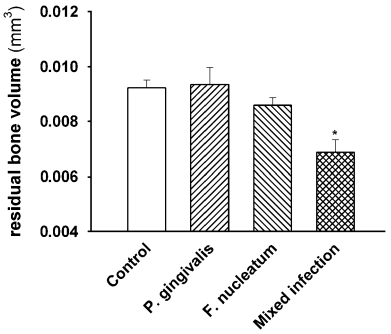

Fig. 2. Evaluation of residual bone loss induced by oral infection with *Porphyromonas gingivalis* (*P. gingivalis*), *Fusobacterium nucleatum* (*F. nucleatum*) or both. Mice ($n = 8$ in each group) were challenged orally three times at 2-day intervals with an innoculum of *P. gingivalis, F. nucleatum* or a mixture of the two. Sham-infected mice served as control. Six weeks later, the jaws were harvested and the alveolar bone volume was measured using micro-computerized tomography. The results are expressed as the mean ± standard error. *, a significant difference from the other groups. $p < 0.05$.

Mixed infection augments the pro-inflammatory cytokine response

To evaluate the early local cytokine response, mice were infected in pre-implanted subcutaneous chambers with *P. gingivalis, F. nucleatum* or both. Saline was injected in the control group.

TNF-α levels

Two hours post-challenge, high levels of TNF-α were induced by infection with *F. nucleatum* or *P. gingivalis/F. nucleatum*, compared with almost undetectable levels in the control group. The TNF-α levels induced by infection with *P. gingivalis* alone were not different from that found in the control mice (Fig. 3a, $p < 0.05$). Although the levels of TNF-α were higher in the *P. gingivalis/F. nucleatum* group than that in the *F. nucleatum* group, the difference was not statistically significant. At 24 h post-infection, there was a decline in TNF-α levels in all the tested groups, with no difference between the mono-infection and the mixed-infection groups.

IL-1β levels

As found for TNF-α, 2 h post-challenge high levels of IL-1β were induced by infection with *F. nucleatum* or *P. gingivalis/F. nucleatum*, compared with almost undetectable levels in the control group. The IL-1β level induced by infection with *P. gingivalis* alone was not different from that found in the control mice, and was significantly lower than that in the other two infected groups (Fig. 3b, $p < 0.05$). At 24 h post-infection this trend was stable, although only the difference between the mixed infection and the *P. gingivalis* groups was statistically significant ($p < 0.05$).

Discussion

Most of the rodent models of experimental periodontis involve oral infection with a single bacterium or ligature placement around a single tooth. Because of the polymicrobial nature of periodontal disease, and the possibility of a synergistic effect between the bacteria, a mouse model of experimental periodontits using more than one bacterium could further our understanding of disease pathogenesis. To test this notion, we focused on a mouse model of mixed infection with two prevalent periodontal pathogens: *P. gingivalis* and *F. nucleatum*. These two bacteria were found to co-aggregate in vitro and in vivo. To test our hypothesis, we compared the clinical outcome of an oral infection with a mixture of the two bacteria to infection with either bacterium alone. Mono-infection with *P. gingivalis* or *F. nucleatum* did not induce significant bone loss compared with that of the control. Although *P. gingivalis* was previously shown to induce bone loss in a similar model (Baker et al. 1994, Wilensky et al. 2005), the bacterial concentration in the previous studies was 10-fold higher than the concentration used in the present one. In addition, more aggressive strains of *P. gingivalis* (ATCC 52977, 381 and W50) were used in the previous studies. Another possible explanation for the lack of differences between the mono-infected and the control groups may be the small sample size of each group ($n = 8$), but this possibility seems less likely. In our animal model, although each bacterium alone did not induce alveolar bone loss, the mixed oral infection, using the same concentration as in the mono-infection groups, resulted in significant bone loss in comparison with that observed in the mono- and sham-infected groups. This could be a result of synergism in viru-

lence between the two bacteria leading to significant induction of periodontal breakdown, or that the bacterial mixture results in greater survival than the monocultures. A study of oral infection in rats showed that mixed infection with *P. gingivalis*, *T. denticola*, *T. forsythia* and *F. nucleatum* induced significant bone loss compared with that following mono-infection with each bacterium alone (Kesavalu et al. 2007). In their study, the addition of *F. nucleatum* to the mixed infection did not change bone resorption. A model using mixed infection with two bacteria may result in different bacterial interactions than mixed infection with four bacteria. Furthermore, we previously showed that the micro-CT technology is more sensitive than the two-dimensional method used by Kesavalu et al. (2007) (Wilensky et al. 2005).

We hypothesized that the dissimilarity in disease expression between mono- and mixed infection is correlated with differences in host response at the molecular level. To test this assumption, we used the subcutaneous chamber model, which enabled us to characterize the inflammatory response to a specific bacterial infection. We are not suggesting that the chamber model is a model for periodontal infection, but a well-established accepted model for quantification of the local host response to bacterial infection. A comparison of the inflammatory response induced by mixed infection *versus* mono-infection showed that the levels of IL-1β and TNF-α were augmented in the mixed-infection group. The difference between the *P. gingivalis* group and the mixed-infection group was significant, whereas the difference between the *F. nucleatum* group and the mixed-infection group was not. The lack of a statistically significant difference between the *F. nucleatum* group and the mixed-infection group might be due to the limited number of animals per group ($n = 6$) used.

IL-1β and TNF-α are two cytokines that are well recognized as tissue-destructive mediators (Bascones et al. 2005), and their high levels in the mixed infection group may explain the results of the oral infection. These findings concur with the results of a previous study that showed that mixed infection with *P. gingivalis* and *F. nucleatum* induces a synergistic destructive effect on soft tissue in the murine abscess model (Feuille et al. 1996). In the same model, tissue destruction continued for 7 days after the bacterial challenge, while the bacterial presence in the infected sites was detectable only up to 2 days post-challenge (Ebersole et al. 1997). This suggests that tissue destruction continues long after the periopathogen infection is eradicated, and that the destruction process is mediated by the host response. It cannot be excluded that the tested cytokine levels found in the *P. gingivalis* mono-infected group and the mixed-infection group may also be affected by the presence of *P. gingivalis*'s proteases. Moreover, it is possible that the presence of *F. nucleatum* along with *P. gingivalis* may interfere to some extent with the proteolysis activity of gingipains and may also affect the detected cytokine levels.

It is generally accepted that periodontal disease is a result of more than one infective organism, albeit the interaction between the pathogenic bacteria is not clear, nor is the impact on disease outcome. The results of the present study clearly show that the mixed infection was more destructive than mono-infection in our mouse model. The nature of the synergistic destruction of the mixed infection may be at least partly attributable to the inter-genera interaction. However, the impact of the interaction between bacteria in a mixed infection on host response merits further investigation. The present study suggests that the oral mixed infection model with *P. gingivalis* and *F. nucleatum* may serve as a rodent model for periodontitis.

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Clinical Relevance

Scientific rationale for the study: Most rodent models for periodontal disease use mono-infection despite the polymicrobial nature of the disease. Our aim was to establish an animal model of poly-microbial infection for the study of periodontal disease pathogenesis, which would better mimic the disease.

Principal findings: Polymicrobial infection with *P. gingivalis* and *F. nucleatum* in mice resulted in augmented alveolar bone loss and a robust inflammatory response, compared with that obtained upon infection with one bacterium alone.

Practical implication: (1) There is a synergistic effect in the induction of experimental periodontitis by *P. gingivalis* and *F. nucleatum*. (2) The *P. gingivalis*/*F. nucleatum* experimental periodontitis model is suitable for future studies of disease pathogenesis and novel prevention approaches.