Oral Microbiology Immunology 2004: 19: 247–251
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Oral Microbiology and Immunology

Modulation of the antibody response by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a mouse model

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Gemmell E, Bird PS, Ford PJ, Ashman RB, Gosling P, Hu Y, Seymour GJ. Modulation of the antibody response by Porphyromonas gingivalis *and* Fusobacterium nucleatum *in a mouse model.*
Oral Microbiol Immunol 2004: 19: 247–251. © Blackwell Munksgaard, 2004.

Successive immunization of mice with *Fusobacterium nucleatum* and *Porphyromonas gingivalis* has been shown to modulate the specific serum IgG responses to these organisms. The aim of this study was to investigate these antibody responses further by examining the IgG subclasses induced as well as the opsonizing properties of the specific antibodies. Serum samples from BALB/c mice immunized with *F. nucleatum* (gp1-F), *P. gingivalis* (gp2-P), *P. gingivalis* followed by *F. nucleatum* (gp3-PF) *F. nucleatum* followed by *P. gingivalis* (gp4-FP) or saline alone (gp5-S) were examined for specific IgG1 (Th2) and IgG2a (Th1) antibody levels using an ELISA and the opsonizing properties measured using a neutrophil chemiluminescence assay. While IgG1 and IgG2a subclasses were induced in all immunized groups, there was a tendency towards an IgG1 response in mice immunized with *P. gingivalis* alone, while immunization with *F. nucleatum* followed by *P. gingivalis* induced significantly higher anti-*P. gingivalis* IgG2a levels than IgG1. The maximum light output due to neutrophil phagocytosis of *P. gingivalis* occurred at 10 min using nonopsonized bacteria. Chemiluminescence was reduced using serum-opsonized *P. gingivalis* and, in particular, sera from *P. gingivalis*-immunized mice (gp2-P), with maximum responses occurring at 40 min. In contrast, phagocytosis of immune serum-opsonized *F. nucleatum* demonstrated peak light output at 10 min, while that of *F. nucleatum* opsonized with sera from saline injected mice (gp5-S) and control nonopsonized bacteria showed peak responses at 40 min. The lowest phagocytic response occurred using gp4-FP serum-opsonized *F. nucleatum*. In conclusion, the results of the present study have demonstrated a systemic Th1/Th2 response in mice immunized with *P. gingivalis* and/or *F. nucleatum* with a trend towards a Th2 response in *P. gingivalis*-immunized mice and a significantly increased anti-*P. gingivalis* IgG2a (Th1) response in mice immunized with *F. nucleatum* prior to *P. gingivalis*. Further, the inhibition of neutrophil phagocytosis of immune serum-opsonized *P. gingivalis* was modulated by the presence of anti-*F. nucleatum* antibodies, while anti-*P. gingivalis* antibodies induced an inhibitory effect on the phagocytic response to *F. nucleatum*.

Key words: *F. nucleatum*; mouse model; opsonization; *P. gingivalis*; specific IgG antibodies

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Accepted for publication March 15, 2004

The infiltrate in the periodontal lesion consists of lymphocytes and macrophages and it has been hypothesized that T lymphocytes predominate in the stable lesion, while the proportion of B cells and plasma cells is increased in the progressive lesion (24, 26, 27). This has prompted the suggestion that T cells with a Th1 cytokine profile may be the major mediator in the

early/stable lesion. The dominance of B cells/plasma cells in the advanced/progressive lesion would suggest a role for Th2 cells (15, 25).

B cells and plasma cells produce and secrete immunoglobulins which protect the host by various methods including prevention of bacterial adherence, inactivation of bacterial toxins and by acting as opsonins for phagocytosis by neutrophils and macrophages. However, specific antibodies do not always eliminate the causative organisms of periodontal disease due to factors such as poor antigenicity of the virulence determinants, elicitation of antibodies with poor antibacterial properties (28) and inhibition of production of specific antibodies (21). Many studies have been reported on specific antibody levels to periodontopathic bacteria such as *Porphyromonas gingivalis*, although the results have been conflicting. While one study showed positive correlations between elevated IgG levels to *P. gingivalis* in both gingivitis associated with puberty and adult periodontitis (22), another study demonstrated no difference in the levels of anti-*P. gingivalis* antibodies in the gingival crevicular fluid of periodontitis patients and healthy control subjects, although there were moderate to strong correlations with serum antibody levels (1). However, the antibody response has been suggested to be generally protective during the chronic phase of the disease (23). Serum from patients with severe periodontitis containing high titers of anti-*P. gingivalis* antibodies completely inhibited *in vitro* bone resorption, whereas serum from patients with low titers failed to inhibit this bone resorption (20). The demonstration of anti-*P. gingivalis* protease antibodies late in periodontitis infections which can block the antiopsonizing activity against C3 and IgG (9) and an increased capacity of serum to opsonize *P. gingivalis* in patients with past destructive periodontal disease (32) also suggest a role for protective antibodies. On the other hand, Cutler et al. (10) reported that only 3/17 serum samples from a number of adult periodontitis patients with elevated IgG to *P. gingivalis* A7436 were opsonic for this particular strain.

Animal models have been used to study the immune responses to periodontopathic bacteria and in recent years, the effects of mixed microbial infections have been reported (4, 11, 34). In the human, *Fusobacterium nucleatum* colonizes the plaque prior to *P. gingivalis* and high levels of *F. nucleatum* have been demonstrated in association with *P. gingivalis* as well as other bacteria associated with periodontal disease, such as *Tannerella forsythia*, *Prevotella intermedia* and *Eikenella corrodens* (31). To investigate the modulatory effects exerted by periodontopathic bacteria on the specific antibody responses, this study examined the specific serum IgG1 (Th2) and IgG2a (Th1) subclass responses and neutrophil phagocytosis of the opsonized organisms after successive immunization of mice with *P. gingivalis* and *F. nucleatum*.

Material and methods

Bacteria

P. gingivalis ATCC 33277 and *F. nucleatum* ATCC 25586 were used in this study. The organisms were cultured anaerobically as described previously by Bird & Seymour (3). Bacteria were harvested from Wilkins Chalgrens agar plates using swabs moistened in reduced normal saline and then suspended in saline. Bacterial numbers for injection were determined using a Helber bacterial counter chamber. The bacteria were suspended in saline in an anaerobic cabinet and then transported in an anaerobic state in tubes with injection caps to the animals to be injected.

Immunization procedure

This project was approved by the institutional animal ethics review committee. BALB/c female mice (6–8 weeks old) were obtained from the University of Queensland Central Animal Breeding House. The immunization protocol has been described previously by Bird et al. (2). Twenty-seven mice were divided into five groups (6/group for groups 1–4 and 3 mice in group 5). Group 1 mice received intraperitoneal injections of 1×10^8 viable *F. nucleatum* in saline once a week for 4 weeks (days 0, 7, 14, and 21). Group 2 mice received 1×10^8 viable *P. gingivalis* organisms in saline as for group 1. Group 3 mice were injected with 1×10^8 viable *P. gingivalis* in saline for the first 2 weeks followed by 1×10^8 viable *F. nucleatum* organisms in saline in weeks 3 and 4. Group 4 mice received the reverse of group 3, with injections of *F. nucleatum* for the first 2 weeks followed by *P. gingivalis* in weeks 3 and 4. Group 5 mice were sham-immunized with one injection of saline per week for 4 weeks. All mice were injected at the same time using the individual protocols for each group.

One week after the final immunizations, the mice from each group were lightly anaesthetized with halothane/O_2 and blood samples collected immediately by heart puncture after which the mice were killed by cervical dislocation. Serum was separated for the determination of specific antibody levels and for opsonization of bacteria in the chemiluminescence assay.

Detection of anti-*F. nucleatum* and *P. gingivalis* antibodies

Serum samples were assayed for the presence of anti-*F. nucleatum* and *P. gingivalis* IgG1 and IgG2a antibodies using an ELISA technique described by Gemmell et al. (14). Briefly, *P. gingivalis* or *F. nucleatum* were coated onto 96-well high-binding plates (Maxisorb Immunoplates, Nunc, Roskilde, Denmark) at a protein concentration of 5.0 μg/ml and after blocking nonspecific sites with 1% bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) in phosphate-buffered saline (PBS)-Tween 20 (0.05%), diluted serum samples were added followed by peroxidase conjugated bovine antimouse IgG1 or IgG2 (at dilutions of 1/2000) (Serotec, Oxford, UK). The substrate containing 0.0075% H_2O_2 and 2.5 mM O'Tolidine (Eastman Kodak, Rochester, NY) was then added and the blue colour reaction stopped after 10 min with 3 M HCl. The optical density of the wells was read at an absorbance of 450 and 655 nm on a Bio-Rad Microplate reader Model 3550. Negative controls consisted of substituting PBS in place of the serum samples.

Levels of anti-*P. gingivalis* and *F. nucleatum* IgG1 and IgG2a antibodies in the serum samples were determined from a standard curve of dilutions of a known concentration of normal mouse IgG (Caltag Laboratories, Burlingame, CA) as described previously (14). Dilutions of the IgG (0.4–50 ng/ml) were coated on to each plate at the same time as the bacterial coating. The ELISA procedure was then followed exactly as described above with the exception of the addition of PBS in place of the mouse serum samples. The levels of specific IgG1 and IgG2a were expressed as being equivalent to μg/ml IgG.

Chemiluminescence assay

Bone marrow cells from BALB/c mice were established using the method of Hart et al. (18). Briefly, femoral and tibial bone marrow from BALB/c mice were collected by washout and passed through a 21-gauge needle to obtain a single cell suspension. The cells were cultured at a concentration

of 2.5×10^6/ml in 75 cm^2 culture flasks (Nunc). In week 3, freshly isolated bone marrow cells were added and after week 4, half of the nonadherent cells were harvested and replaced with fresh medium weekly resulting in continuous cell cultures.

The chemiluminescence assay was performed as described by Wright et al. (33). Nonadherent neutrophils were harvested from the bone marrow cell cultures as described by Hart et al. (18), fractionated using discontinuous Percoll density gradients and then suspended in PBS supplemented with Ca^{2+} and Mg^{2+}. The assay used 96-well opaque white plates (Nunc) which were blocked overnight at 4°C with 1% bovine serum albumin (Commonwealth Serum Laboratories). Approximately 2.5×10^7 *P. gingivalis* and *F. nucleatum* were opsonized with the test serum samples for 30 min at 37°C, washed and resuspended in Ca^{2+} and Mg^{2+} free PBS and added to each well together with 3 mmol Luminol in PBS (Sigma Aldrich, St Louis, MO). Neutrophils were finally added at a concentration of 2.5×10^5/well. Controls used nonopsonized bacteria in PBS. Chemiluminescence was measured using a 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, Turku, Finland) by light output integrated over 10-s intervals. The counts per second were read at 10, 40, 70, and 100 min, plates being incubated at 37°C between readings.

Statistical analysis

Multivariate analysis of variance using the general linear model was used to test for differences in the levels of specific anti-IgG1 and IgG2a *P. gingivalis* and *F. nucleatum* antibodies and neutrophil chemiluminescence within and between each of the five groups. Selected pairs of groups were then tested for significance using the Student's *t*-test. The Minitab statistical package (Minitab Inc., State College, PA) was used to perform the analyses.

Results

Serum specific antibody responses

Anti-*P. gingivalis* IgG1 and IgG2a antibody levels in gp2-P mice were lower than the respective levels in gp3-PF mice ($P < 0.023$) (Fig. 1A, B). Anti-*P. gingivalis* IgG1 antibodies were not demonstrated in gp4-FP mice.

Anti-*F. nucleatum* IgG1 and IgG2a antibody levels were increased in gp1-F and gp4-FP compared with those in gp2-P and in the case of IgG1 levels, higher than those in gp3-PF ($P < 0.031$) (Fig. 1A, B).

In gp4-FP mice immunized with *F. nucleatum* followed by *P. gingivalis*, the anti-*P. gingivalis* IgG2a antibody response was higher than that of the IgG1 response ($P = 0.008$).

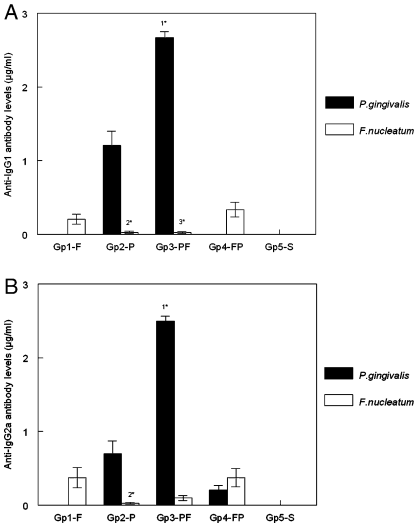

Fig. 1. The mean µg/ml (± standard error of the mean) specific anti-*P. gingivalis and* anti-*F. nucleatum* IgG1 (A) and IgG2a (B) antibody levels in the serum samples of BALB/c mice immunized with *F. nucleatum* (gp1-F), *P. gingivalis* (gp2-P), *P. gingivalis* followed by *F. nucleatum* (gp3-PF), *F. nucleatum* followed by *P. gingivalis* (gp4-FP) and saline (gp5-S). **1***. Anti-*P. gingivalis* IgG1 and IgG2a antibody levels were higher in gp3-PF compared with gp2-P ($P < 0.023$). **2***. Anti-*F. nucleatum* IgG1 and IgG2a antibody levels were decreased in gp2-P compared with gp1-F and gp4-FP ($P < 0.031$). **3***. Anti-*F. nucleatum* IgG1 levels were decreased in gp3-PF compared with gp1-F and gp4-FP ($P < 0.025$).

Neutrophil chemiluminescence

Phagocytosis of nonopsonized *P. gingivalis* resulted in a peak light output at 10 min, which was significantly higher than that of the serum opsonized *P. gingivalis* (groups 1–5) ($P < 0.035$). Chemiluminescence had reduced significantly by 70 min ($P < 0.045$) and 100 min ($P < 0.016$) compared with that of *P. gingivalis* opsonized with immune serum from groups 1–4 (Fig. 2A).

Phagocytic responses using *P. gingivalis* opsonized with serum from groups 1–5 peaked at 40 min. Chemiluminescence using serum samples from saline immunized gp5-S mice was significantly higher than gp1-F, gp2-P and gp3-PF ($P < 0.029$) while that of gp2-P mice was lower than that of gp4-FP ($P = 0.011$) (Fig. 2A).

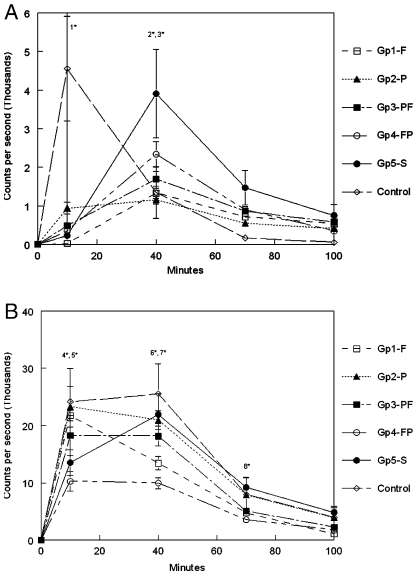

Fig. 2. The mean counts per second (± standard error of the mean) chemiluminescence due to neutrophil phagocytosis of *P. gingivalis* (A) and *F. nucleatum* (B) opsonized with serum samples from BALB/c mice immunized with *F. nucleatum* (gp1-F), *P. gingivalis* (gp2-P), *P. gingivalis* followed by *F. nucleatum* (gp3-PF), *F. nucleatum* followed by *P. gingivalis* (gp4-FP), saline (gp5-S) and finally control nonopsonized organisms. **1***. Chemiluminescence of nonopsonized *P. gingivalis* (control) was higher than that of all serum opsonized *P. gingivalis* (gps 1–5) at 10 min ($P < 0.035$). **2***. Chemiluminescence using gp5-S sera was higher than gp1-F, gp2-P and gp3-PF at 40 min ($P < 0.029$). **3***. Chemiluminescence using gp2-P sera was lower than that of gp4-FP at 40 min ($P = 0.011$). **4***. Chemiluminescence using gp1-F serum opsonized *F. nucleatum* was higher than that using gp4-FP and gp5-S serum samples at 10 min ($P < 0.006$). **5***. Chemiluminescence using gp4-FP sera was lower than that using gp2-P serum and control nonopsonized bacteria at 10 min ($P < 034$). **6***. Chemiluminescence of control nonopsonized *F. nucleatum* was higher than that of gp1-F and gp4-FP at 40 min ($P < 0.012$). **7***. Chemiluminescence using gp4-FP sera was lower at 40 min compared with gp3-PF and gp5-S ($P < 0.019$). **8***. Chemiluminescence using gp4-FP sera was lower at 70 min compared with gp2-P and gp5-S ($P < 0.018$).

Phagocytosis of *F. nucleatum* opsonized with sera from saline injected gp5-S mice and control nonopsonized bacteria showed peak responses at 40 min, the latter response being higher than those of gp1-F and gp4-FP ($P < 0.012$) (Fig. 2B).

Neutrophil phagocytosis of *F. nucleatum* opsonized with serum from groups 1–4 demonstrated peak light output at 10 min, responses using sera from gp1-F *F. nucleatum* immunized mice being significantly higher than those using gp4-FP

and gp5-S serum samples ($P < 0.006$) (Fig. 2B). The response of gp4-FP serum opsonized *F. nucleatum* was significantly lower than that using gp2-P serum and control nonopsonized bacteria ($P < 0.034$) and remained lower at 40 min compared with gp3-PF and gp5-S ($P < 0.019$), and at 70 min compared with gp2-P and gp5-S ($P < 0.018$) (Fig. 2B).

Discussion

The results of the present study have demonstrated the production of both IgG1 and IgG2a subclass antibodies in response to immunization with *P. gingivalis* or *F. nucleatum*. Mice immunized with *P. gingivalis* prior to *F. nucleatum* (gp3-PF) demonstrated a significant increase in the levels of both anti-*P. gingivalis* IgG1 and IgG2a antibodies compared with group 2 mice immunized with *P. gingivalis* only. As the total IgG antibody levels in the former group were shown to be reduced in comparison with the latter group (14), the results suggest a selective enhancement of these two subclasses by *F. nucleatum*.

The results showed a trend towards the production of specific IgG1 antibodies in response to *P. gingivalis* immunization only, while injection with *F. nucleatum* followed by *P. gingivalis* induced a significant increase in anti-*P. gingivalis* IgG2a levels. A decrease in anti-*F. nucleatum* IgG1 antibodies in group 3 (immunized with *P. gingivalis* prior to *F. nucleatum*) was also demonstrated. The Th1 cytokine IFN-γ enhances IgG2a antibodies (13, 30) while the Th2 cytokine interleukin (IL)-4 enhances IgG1 in mice (8, 29). The results indicate therefore that *F. nucleatum* has induced a switch in *P. gingivalis* antibodies towards a Th1 response. We have demonstrated previously that immunization with *P. gingivalis* induces a Th1/Th2 cytokine profile in the spleens of immunized mice (17), immunization with both *P. gingivalis and F. nucleatum* inducing an increase in the percentage of splenic IL-4^+ and IFN-γ^+ T cells compared with responses to either bacterium alone (14). However, an immunohistologic study showed a tendency towards Th2 IgG1+ plasma cells in the gingival tissues of *P. gingivalis*-induced subcutaneous lesions of BALB/c mice (16). In contrast to these studies, Choi et al. (7) reported that T-cell clones derived from the spleens of BALB/c mice immunized with *F. nucleatum* followed by *P. gingivalis* had an IL-4 Th2 profile, whereas those established from mice immunized with *P. gingivalis* only demonstrated an IFN-γ Th1 profile. Further, Herminajeng et al. (19) found that IL-10 depletion in BALB/c mice prior to immunization with *P. gingivalis* lipopolysaccharide, significantly reduced the serum anti-*P. gingivalis* lipopolysaccharide IgG1 and increased the IgG2a response. However, we have demonstrated an increased percentage of IL-10^+ T cells rather than a decrease in response to immunization with both *P. gingivalis and F. nucleatum* (14), further suggesting the role of IFN-γ in steering the response towards a Th1 response.

Chemiluminescence is the light energy produced by neutrophils during interaction with bacteria and reflects the oxidative metabolic response following initiation of phagocytosis (5). The results of the present study demonstrated that peak luminescence of neutrophil phagocytosis of *F. nucleatum* opsonized with immune serum occurred at 10 min, whereas that of *F. nucleatum* opsonized with nonimmune serum or of nonopsonized *F. nucleatum* was delayed until 40 min, indicating an enhanced opsonic effect by anti-*F. nucleatum* antibodies. Opsonization of *F. nucleatum* using gp4-FP serum samples containing both anti-*P. gingivalis and* anti-*F. nucleatum* antibodies induced the lowest phagocytic response. In contrast to *F. nucleatum*, phagocytosis of *P. gingivalis* was inhibited. The peak response to serum-opsonized *P. gingivalis* was reduced and occurred at 40 min, although the response using nonimmune serum showed the least inhibiton. Of the immune serum samples, those from group 4 mice immunized with *F. nucleatum* prior to *P. gingivalis* (gp4-FP) induced the least inhibitory response, indicating a modulatory effect by anti-*F. nucleatum* antibodies. Therefore, while anti-*F. nucleatum* antibodies reduced the inhibitory effect of the phagocytic response to *P. gingivalis*, anti-*P. gingivalis* antibodies appeared to induce an increased inhibitory response to *F. nucleatum*. These results are in contrast to the study by Choi et al. (6), which showed that serum samples from BALB/c mice immunized with *F. nucleatum* prior to *P. gingivalis* reduced the opsonophagocytosis function against *P. gingivalis*. The results of the present study also demonstrated much higher levels of chemiluminescence during phagocytosis of *F. nucleatum* compared with *P. gingivalis*. Whether this is due to the inhibitory effect of *P. gingivalis* or the black pigmentation interfered with light output is not known, although immunization with both bacteria resulted in lower chemiluminescence.

In the human, neutrophils from patients with rapidly progressive periodontitis have been demonstrated to have deficiencies in phagocytotic activity and in the intracellular killing of *P. gingivalis* compared with cells from healthy subjects (12). However, an increased capacity of serum to opsonize *P. gingivalis* has been shown to be a distinctive feature in patients with past destructive periodontal disease (32). Furthermore, anti-*P. gingivalis* protease antibodies which occur late in periodontitis infections have been demonstrated to block the antiopsonizing activity against C3 and IgG (9). It may be that these *P. gingivalis* proteases are responsible for the reduced chemiluminescence demonstrated in the present study and they may play a major role in resistance to phagocytosis.

In conclusion, the results of the present study have demonstrated a Th1/Th2 response in mice after immunization with *P. gingivalis and F. nucleatum* with a trend towards a Th2 response in *P. gingivalis*-immunized mice. However, when mice were immunized with *F. nucleatum* prior to *P. gingivalis* there was a significantly increased IgG2a (Th1) response to *P. gingivalis*. Further, the inhibition of neutrophil phagocytosis of immune serum-opsonized *P. gingivalis* was modulated by the presence of anti-*F. nucleatum* antibodies, while anti-*P. gingivalis* antibodies induced an inhibitory effect on the phagocytic response to *F. nucleatum*.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia.

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