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Immunohistological analysis of *Tannerella forsythia*-induced lesions in a murine model

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Tannerella forsythia has been implicated as a defined periodontal pathogen. In the present study a mouse model was used to determine the phenotype of leukocytes in the lesions induced by subcutaneous injections of either live (group A) or nonviable (group B) *T. forsythia.* Control mice (group C) received the vehicle only. Lesions were excised at days 1, 2, 4, and 7. An avidin-biotin immunoperoxidase method was used to stain infiltrating $CD4^+$ and $CD8^+$ T cells, $CD14^+$ macrophages, $CD19^+$ B cells, and neutrophils. Hematoxylin and eosin sections demonstrated lesions with central necrotic cores surrounded by neutrophils, macrophages and lymphocytes in both group A and group B mice. Lesions from control mice exhibited no or only occasional solitary leukocytes. In both groups A and B, neutrophils were the dominant leukocyte in the lesion 1 day after injection, the numbers decreasing over the 7-day experimental period. There was a relatively low mean percent of $CD4^+$ and $CD8^+$ T cells in the lesions and, whereas the percent of $CD8^+$ T cells remained constant, there was a significant increase in the percent of $CD4^+$ T cells at day 7. This increase was more evident in group A mice. The mean percent of $CD14^+$ macrophages and $CD19^+$ B cells remained low over the experimental period, although there was a significantly higher mean percent of $CD19^+$ B cells at day 1. In conclusion, the results showed that immunization of mice with live *T. forsythia* induced a stronger immune response than nonviable organisms. The inflammatory response presented as a nonspecific immune response with evidence of an adaptive (T-cell) response by day 7. Unlike *Porphyromonas gingivalis*, there was no inhibition of neutrophil migration.

Key words: immunohistology; leukocytes; mouse model; *Tannerella forsythia*

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Chronic inflammatory periodontal disease and the loss of attachment associated with periodontal disease result from the host's inflammatory response to bacteria present in dental plaque. *Tannerella forsythia, Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* are considered to be key pathogens associated with periodontal disease in humans (32).

Neutrophils migrate through the junctional epithelium and into the gingival sulcus, where they are the major leukocyte. Neutrophils can minimize the destructive effects of plaque bacteria (5); however, it has been shown that bacteria such as *P. gingivalis* are able to evade host detection and innate immune responses (4). In the connective tissues, the inflammatory infiltrate consists predominantly of lymphocytes (T cells) and macrophages (23, 24). If the adaptive response is unable to contain the microbial challenge, a B-cell response may develop, resulting in protective antibody and subsequent control of the infection, or nonprotective antibodies and connective tissue destruction and bone loss (19). Activated macrophages are essential for innate resistance to intracellular infection. Macrophages are important mediators of inflammation in the connective tissue infiltrate, where they produce cytokines and also present antigens to T cells (5). Complete elimination of pathogens, however, involves antigen-specific T and B cells and the interdependence of the innate and adaptive immune systems (28).

Many studies have reported on various aspects of *P. gingivalis* and *A. actinomycetemcomitans* infection. These studies have mainly focused on the detection of

organisms and specific antibody levels in healthy and periodontitis subjects (12, 25). Studies on *T. forsythia* have shown that the organism is found in sites of periodontal breakdown in higher numbers than in sites of gingivitis or health (14, 17, 20). *T. forsythia* has also been detected more frequently and in higher numbers in active periodontal lesions than in inactive lesions (6). Other reports have showed that subjects who harbored *T. forsythia* were at greater risk of attachment loss than were subjects in whom *T. forsythia* was not detected (13, 18). Unlike *P. gingivalis* and *A. actinomycetemcomitans*, few studies have been reported on the immune response to *T. forsythia.*

Animal models have been used widely to obtain a clearer picture of the pathogenic potential of periodontopathogens such as *P. gingivalis*. They have provided important information for building an understanding of responses to periodontopathic bacteria, either to individual organisms or to mixed infections. The pathogenicity of *P. gingivalis* has been established in both primate and rodent models. The mouse abscess model in particular has been used to demonstrate that immunization with whole organisms or extracts of *P. gingivalis* results in the development of protection with fewer secondary lesions and no septicemia (1, 3, 11, 15).

The aim of this study was to use the mouse abscess model to study the pathogenicity of *T. forsythia*. The effects of a subcutaneous injection with either live or nonviable *T. forsythia* were determined with reference to infiltrating $CD4^+$ and $CD8^+$ T cells, $CD14^+$ macrophages, $CD19^+$ B cells, and neutrophils.

Material and methods

Bacteria preparation

T. forsythia ATCC 43037™ was cultured anaerobically and prepared for injection as described by Bird et al. (2).

Mouse strain

This project was approved by the institutional animal ethics review committee. Female BALB/c mice (6–8 weeks old) were obtained from the University of Queensland Central Animal Breeding House.

Immunization procedure

Thirty-two mice were used in this study. Each mouse was given subcutaneous injections into two sites on either side of the shaved dorsal surface, approximately 1 cm either side of the midline as described previously (2). The mice were divided into three groups. Group A mice (12 mice) received an injection of live *T. forsythia* organisms (1×10^9 cells/site) in phosphate buffered saline (pH 7.2) and 15 mg/l N-acetyl muramic acid (PBS-NAM). Group B (12 mice) were immunized with heat-killed *T. forsythia* (1×10^9 cells/site) in PBS-NAM. Control group C (8 mice) were given PBS-NAM only.

Three mice from groups A and B, and two mice from group C were killed on days 1, 2, 4, and 7. The mice from each group were lightly anesthetized with carbon dioxide and killed by cervical dislocation as described previously (10). Immediately after death the lesions from all mice were carefully excised, embedded in OCT, quenched and then stored in liquid nitrogen. Serial cryostat sections of 6 μm were cut from each specimen, air dried for 2 h, fixed in equal parts chloroform/acetone for 5 min and stored at −20°C (21).

Immunoperoxidase technique

$CD4^+$ and $CD8^+$ T-cell subsets, $CD14^+$ cells (activated monocytes/macrophages and dendritic cells but not unstimulated macrophages, dendritic cells, neutrophils or blood monocytes) and $CD19^+$ B cells (but not terminally differentiated plasma cells) were labeled using an avidin-biotin immunoperoxidase method described previously (8, 9). Briefly, after rehydration in PBS pH 7.2, the sections were incubated with rat anti-mouse CD4 (L3T4), CD8a (Ly-2), CD14 or CD19 (Pharmingen, San Diego, CA). All antibodies were used at dilutions of 1/20. This step was followed by biotinylated rabbit anti-rat immunoglobulins (DAKO, Glostrup, Denmark) and finally strepavidin peroxidase (DAKO), both at dilutions of 1/50. The peroxidase was developed using the DAKO liquid DAB Substrate-Chromogen system (DAKO). Nuclei were counterstained with Mayer's hematoxylin.

Peroxidase-positive neutrophils were stained using the DAB kit as described above for the immunoperoxidase technique followed by counterstaining with Mayer's hematoxylin.

Mouse spleen sections were prepared in the same way as the lesions and used as positive controls. Negative controls included the use of PBS in place of the primary antibody.

Cell analysis

Qualitative assessment of each lesion was undertaken using hematoxylin & eosin-stained sections. For the immunoperoxidase-stained sections, high power fields (×400) of each lesion were viewed and three or more representative fields were counted using a graticule. The mean percent (± standard error of the mean) of positive $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ cells and peroxidase-positive neutrophils were determined as a percentage of the total number of cells in each infiltrate by counting the total number of infiltrating cells within each field and the total numbers with positive membrane staining.

Statistical analysis

Multivariate analysis of variance using the general linear model was used to test for differences between the percentages of $CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ cells and neutrophils in the lesions. Comparisons were made within each group and between the sham-immunized control group C and group A mice immunized with live *T. forsythia* and group B mice immunized with nonviable *T. forsythia* at each time interval. Pairs of groups were then tested for significance using Student's *t*-test. A significance level of 0.03 was determined to reduce the probability of significant differences occurring by chance. The Minitab statistical package (Minitab Inc., State College, PA) was used to perform the analyses.

Results

The hematoxylin and eosin-stained sections showed that the lesions in group A and group B mice were granulomatous tissue responses with large central cores (Fig. 1A, B). Peroxidase-positive granules were detected in the core and, although intact peroxidase positive neutrophils were evident, the granules were most likely due to degranulation of neutrophils (Fig. 1A). The presence of macrophages in the core was indicated by CD14 staining. The control group C did not demonstrate lesions (Fig. 1C).

Intact neutrophils were present in the area surrounding the core containing capillaries (Fig. 2A), alongside lymphocytes (Fig. 2B–D) and macrophages (Fig. 2E) as determined by T-cell, B-cell and macrophage staining. The $CD14^+$ cells demonstrated the morphologic appearance of macrophages rather than dendritic cells. Counting of cell populations was performed in this area.

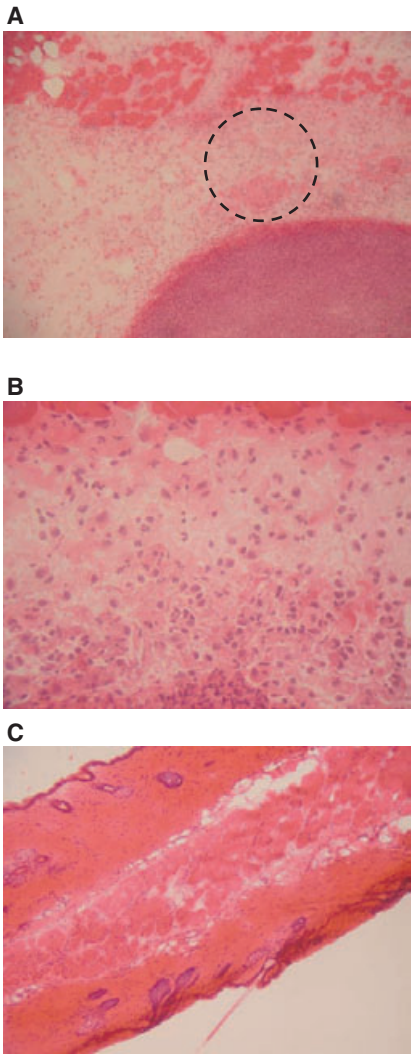
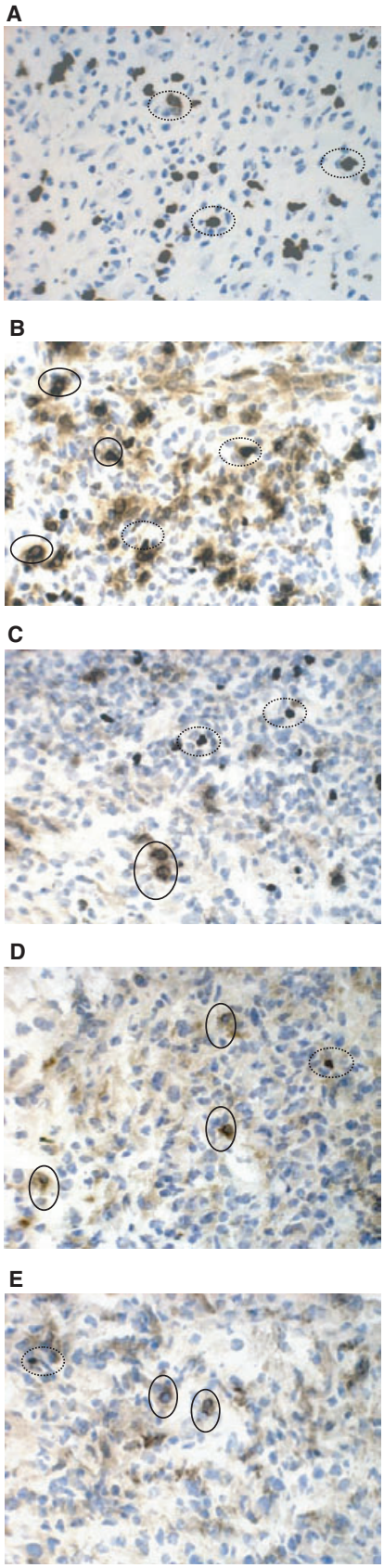


Fig. 1. A) H&E section showing the area surrounding the core of a lesion from a BALB/c mouse immunized with live *T. forsythia* (original magnification × 10). Dashed circle represents area represented in (B). B) Higher powered view of the same H&E section as in (A) showing the region where infiltrating leukocytes were counted (original magnification × 40). C) No evidence of lesion formation in this H&E section of the injection site from a control group C mouse injected only with PBS-NAM (original magnification × 10).

Neutrophils

The mean percent of neutrophils was highest in the lesions at day 1 in both groups A and B, and while the percent of positive cells was higher in group B than in group A, these results were not significant at the 0.03 level. The percent of positive neutrophils decreased in group B at day 2 ($P = 0.018$) and group A at day 4 ($P = 0.031$) although this was not quite significant at the 0.03 level. The percent of positive cells was significantly higher in group A than in control group C at days 1, 2, and 7 ($P < 0.027$) and in group B compared with control group C at day 1 ($P = 0.029$) (Fig. 3A).



CD4$^+$ T cells

Very small percentages of CD4$^+$ T cells were noted at day 1. There was a significant increase in group A at day 7 compared with days 1, 2, and 4 ($P < 0.023$) and in group B compared with days 2 and 4 ($P < 0.028$). The percent of positive cells was higher than the control group C at day 1 ($P = 0.031$), being not quite significant at the 0.03 level (Fig. 3B).

CD8$^+$ T cells

Again, very low percentages of CD8$^+$ cells were present in the lesions at day 1. In group A mice, there was a decrease at day 7 compared with day 1 ($P = 0.037$) although again this was not quite significant at the 0.03 level. In group B mice immunized with nonviable bacteria, there was an increase at day 4 compared to day 2 ($P < 0.028$). The percent of positive cells was significantly higher at day 1 in group A than in the control group C, and in group B than in the control group ($P < 0.016$) (Fig. 3C).

CD19$^+$ B cells

Like the T cells, very low percentages of CD19$^+$ were present in the lesions of group A and B mice at day 1, decreasing further over the 7-day period (Fig. 3D). In group A mice immunized with viable bacteria, this decrease was significant at days 4 and 7 compared with day 1 ($P < 0.010$) and in group B, significantly lower on day 2 than day 1 ($P = 0.017$). At day 1 the percent of CD19$^+$ cells in

Fig. 2. A) Peroxidase positive granules in the area surrounding the core of a lesion from a BALB/c mouse injected with nonviable *T. forsythia* (original magnification × 40). Neutrophils – hatched circles. B) Numerous CD4$^+$ T cells in the area surrounding the core of lesion from a BALB/c mouse immunized with live *T. forsythia* (original magnification × 40). CD4$^+$ T cells – solid circles; neutrophils – hatched circles. C) Few CD8$^+$ T cells in the area surrounding the core of lesion from a BALB/c mouse immunized with live *T. forsythia* (original magnification × 40). CD8$^+$ T cells – solid circles; neutrophils – hatched circles. D) Few CD19$^+$ B cells in the area surrounding the core of lesion from a BALB/c mouse immunized with nonviable *T. forsythia* (original magnification × 40). CD19$^+$ B cells – solid circles; neutrophils – hatched circle. E) Few CD14$^+$ macrophages in the area surrounding the core of lesion from a BALB/c mouse immunized with live *T. forsythia* (original magnification × 40). CD14$^+$ macrophages – solid circles; neutrophils – hatched circle.

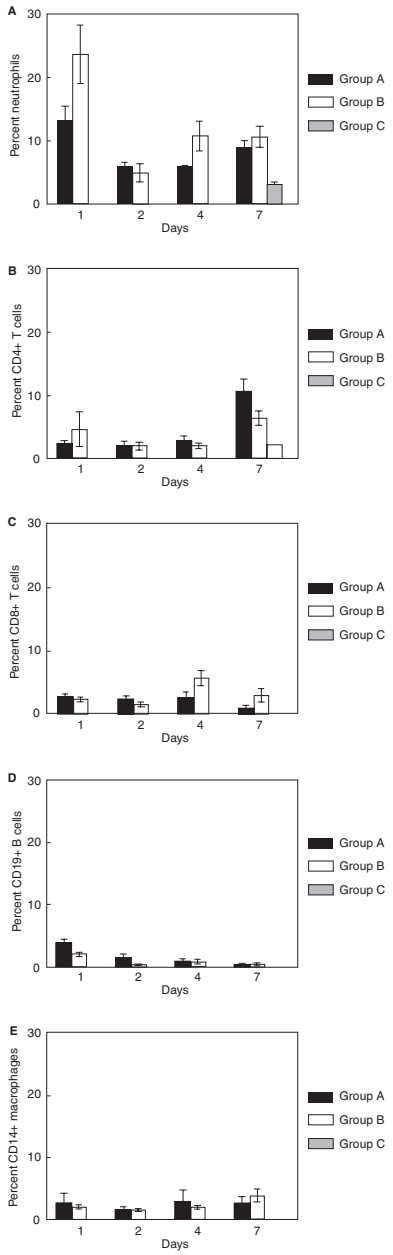


Fig. 3. The mean percent of (± standard error of the mean) neutrophils (A), $CD4^+$ T cells (B), $CD8^+$ T cells (C), $CD19^+$ B cells (D) and $CD14^+$ macrophages (E) in the lesions of BALB/c mice immunized with *T. forsythia* (group A), nonviable *T. forsythia* (group B) and PBS-NAM only (control group C) after 1, 2, 4, and 7 days.

group A was significantly greater than in control group C ($P = 0.008$); in group B the percent of positive cells was higher at day 2 than in group C ($P = 0.011$) (Fig. 3D).

$CD14^+$ macrophages

$CD14^+$ cells had the morphology of macrophages, $CD14^+$ dendritic cells not being observed. Low percentages of $CD14^+$ positive cells were present over the 7-day period. The percent of positive cells in group B was significantly higher than the control group C at days 1 and 4 ($P < 0.017$) (Fig. 3E).

Comparisons between cell subsets

Neutrophils were the predominant leukocyte at day 1 in groups A and B. The percent of positive neutrophils in group A lesions was higher than the percent of $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ cells at days 1 ($P < 0.028$) and 2 ($P < 0.017$), $CD8^+$ T cells and $CD19^+$ B cells at day 4 ($P < 0.024$) and $CD8^+$, $CD19^+$ and $CD14^+$ cells at day 7 ($P < 0.014$). The percent of positive neutrophils in group B lesions was higher than the other four cell types at day 1 ($P < 0.025$), $CD4^+$ T cells and $CD19^+$ B cells at day 4 ($P < 0.023$) and $CD8^+$, $CD19^+$ and $CD14^+$ cells at day 7 ($P < 0.027$) (Fig. 3).

In group A lesions at day 7, the mean percent of $CD4^+$ cells was higher than that of $CD8^+$, $CD14^+$ and $CD19^+$ cells ($P < 0.024$). In group B lesions, the percent of $CD4^+$ cells was higher than $CD19^+$ cells at day 2 ($P = 0.007$). The percent of $CD8^+$ cells was higher $CD19^+$ cells at day 4 ($P < 0.016$). The percent of $CD14^+$ cells was increased in comparison with $CD19^+$ cells at day 7 ($P = 0.032$), although again this was not quite significant at the 0.03 level (Fig. 3).

Discussion

The results of the present study have shown that mice injected with subcutaneous injections of live and nonviable *T. forsythia,* developed lesions consisting of a granulomatous tissue response with a central large core as described previously for *P. gingivalis*-induced lesions (1). The control group, which received injections of PBS-NAM only, showed very little evidence of an immune response. The response in *T. forsythia*-injected mice consisted primarily of an innate immune response, neutrophils being the dominant leukocyte 1 day after injection. There was a stronger neutrophil response in group B mice injected with nonviable *T. forsythia* than in group A mice immunized with live organisms, although this result was not significant. Bird et al. (2) showed that BALB/c mice immunized subcutaneously with live *T. forsythia* had a stronger response in terms of lesion size from day 1 than mice immunized with nonviable *T. forsythia.* It is therefore possible that the peak in neutrophils numbers occurred earlier than 24 h in group A mice. *P. gingivalis* has been shown to inhibit the migration of neutrophils into *P. gingivalis* induced lesions. These mice were immunized with PBS only prior to receiving subcutaneous injections of *P. gingivalis,* the experimental protocol being similar to that of the present study. Mice immunized previously with *P. gingivalis* did not show neutrophil inhibition, possibly because of the production of specific anti-*P. gingivalis* antibodies which most likely possessed opsonic properties (8). Unlike *P. gingivalis,* the present study has shown that *T. forsythia* caused no inhibition of neutrophils and therefore no evasion of the innate host defence mechanism.

The results of the present study also showed a relatively low percentage of T cells in the lesions. Although the percent of $CD8^+$ T cells remained constant, there was a significant increase in the percent of $CD4^+$ T cells at day 7, the increase in migration being more evident in group A mice immunized with live bacteria. Bird et al. (2) showed that anti-*T. forsythia* antibody levels in the serum samples from mice immunized with live *T. forsythia* remained stable from days 1–4 and then rose significantly at day 7. *T. forsythia*-specific helper T cells are most likely necessary for the production of anti-*T. forsythia* antibodies, so the increase in $CD4^+$ T cells in the *T. forsythia*-induced lesions may have been the result of migration of *T. forsythia*-specific T cells. The slower kinetic response in group B mice immunized with nonviable *T. forsythia* probably relates to the lower levels of anti-*T. forsythia*-specific antibodies induced in these mice (2).

Although there were few $CD19^+$ B cells in the lesions, there was a significantly higher percentage at day 1. As stated above, specific anti-*T. forsythia* antibody levels rose significantly by day 7 after immunization (2) so that the higher percent of $CD19^+$ B cells at day 1 represents a nonspecific response. Gemmell et al. (9) also showed that the percent of $CD19^+$ B cells was very low in *P. gingivalis*-induced lesions. However, numerous plasma cells were demonstrated primarily towards the periphery of the lesions. As these mice were immunized three times prior to being challenged with subcutaneous injections of *P. gingivalis,* the plasma cells in the *P. gingivalis*-induced lesions most likely reflect a specific secondary adaptive immune response.

Macrophages were also present in low numbers for the 7-day experimental per-

iod. In *P. gingivalis*-induced lesions (8), acid phosphatase positive (AP+) and nonspecific esterase-positive (NSE+) macrophages were not detected until day 4; by day 7 these were found mainly on the periphery of the lesion in mice immunized previously with *P. gingivalis* and were associated with healing lesions. In control mice, which received subcutaneous injections of *P. gingivalis* only, macrophages became the dominant cell type by day 14. The study by Bird et al. (2) showed that by day 9, *T. forsythia*-induced lesion sizes in immunized mice had not yet reduced in size, indicating that healing would probably not occur until at least day 14.

Although the results of the present study have shown that *T. forsythia* may not have the same pathogenic effects as *P. gingivalis,* Socransky et al. (26) suggested the virulence of *T. forsythia* may occur when in association with other periodontopathogens. These authors found that cocultivation of *T. forsythia* with other periodontopathogens accelerated the growth of *T. forsythia*, possibly by providing necessary peptides. Further, Takemoto et al. (27) found that coinfection of *P. gingivalis* and *T. forsythia* in a rabbit abscess model induced lesions, sometimes resulting in death, whereas no lesions were induced following mono-infection.

Feuille et al. (7) reported on the synergistic effect of *P. gingivalis* and *F. nucleatum* in the murine abscess model and found that this was partly due to the proteolytic activity of *P. gingivalis.* Yoneda et al. (31) showed that the gingipains of *P. gingivalis* played an important role in the pathological synergy between *T. forsythia* and *P. gingivalis*. Further, Sabet et al. (22) showed that the S-layer, an antigenic layer outside the outer membrane of *T. forsythia*, is involved in the infectious process of abscess formation in mice. This study observed through antibody inhibition assays that the adherent/invasive activities of *T. forsythia* were mediated by the S-layer. Another study by Yoneda et al. (30) in humans found the IgG responses against the *T. forsythia* S-layer were low in healthy control subjects, but were significantly higher in subjects with periodontal disease. Mice immunized with the purified S-layer and whole *T. forsythia* and then challenged with live *T. forsythia* did not develop abscesses. Mice that had not been immunized developed abscesses after challenge with *T. forsythia*, suggesting that the immune response to the S-layer of *T. forsythia* is protective.

In conclusion, the present immunohistological study has shown that immunization of mice with live *T. forsythia* induced a stronger immune response than immunization with nonviable organisms. The inflammatory response presented as a nonspecific response with evidence of an adaptive (T-cell) response by day 7. Unlike *P. gingivalis, T. forsythia* did not inhibit neutrophil migration. *T. forsythia* as well as *P. gingivalis* have been reported to be the strongest bacterial markers for periodontal disease progression and are infrequently cultured from subjects without periodontal bone loss (16, 29). Studies are necessary to delineate further the response to these periodontopathogens.

Acknowledgment

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References

1. Bird PS, Gemmell E, Polak B, Paton RG, Sosroseno W, Seymour GJ. Protective immunity to *Porphyromonas gingivalis* infection in a murine model. J Periodontol 1995: **66**: 351–362.
2. Bird PS, Shakibaie F, Gemmell E, Polak B, Seymour GJ. Immune response to *Bacteroides forsythus* in a murine model. Oral Microbiol Immunol 2001: **16**: 311–315.
3. Chen PB, Davern LB, Schifferle R, Zambon JJ. Protective immunization against experimental *Bacteroides (Porphyromonas) gingivalis* infection. Infect Immun 1990: **58**: 3394–3400.
4. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. Periodontol 2000 1997: **14**: 54–78.
5. Dennison DK, Van Dyke TE. The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. Periodontol 2000 1997: **14**: 12–32.
6. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 1988: **15**: 316–323.
7. Feuille F, Ebersole JL, Kesavalu L, Steffen M, Holt S. Mixed infection with *Porphyromonas gingivalis* and *Fusobacteria nucleatum* in a murine lesion model: potential synergistic effects on virulence. Infect Immun 1996: **64**: 2095–2100.
8. Gemmell E, Bird PS, Bowman JD. Immunohistological study of *Porphyromonas gingivalis*-induced lesions in a murine model. Oral Microbiol Immunol 1997: **12**: 288–297.
9. Gemmell E, Winning TA, Carter CL, Ford PJ, Bird PS, Ashman RB, et al. Differences in mouse strain influence leukocyte and immunglobulin phenotype response to *Porphyromonas gingivalis*. Oral Microbiol Immunol 2003: **18**: 364–370.
10. Gemmell E, Winning TA, Grieco DA, Bird PS, Seymour GJ. The influence of genetic variation on the splenic T cell cytokine and specific serum antibody response to *Porphyromonas gingivalis* in mice. J Periodontol 2000: **71**: 1130.
11. Genco CA, Kapczynski DR, Cutler CW, Arko RJ, Arnold RR. Influence of immunization on *Porphyromonas gingivalis* colonization and invasion in the mouse chamber model. Infect Immun 1992: **60**: 1447–1454.
12. Hamlet SM, Cullinan MP, Westerman B, Lindeman M, Bird PS, Palmer J, et al. Distribution of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. J Clin Periodontol 2001: **28**: 1163–1171.
13. Hamlet SM, Ellwood R, Cullinan MP, Worthington H, Palmer J, Bird PS, et al. Persistent colonization with *Tannerella forsythensis* and loss of attachment in adolescents. J Dent Res 2004: **83**: 232–235.
14. Herrera D, Roldan S, Gonzalez I, Sanz M. The periodontal abscess (1). Clinical and microbiological findings. J Clin Periodontol 2000: **27**: 387–394.
15. Kesavalu L, Ebersole JL, Machen RL, Holt SC. *Porphyromonas gingivalis* virulence in mice: induction of immunity to bacterial components. Infect Immunol 1992: **60**: 1455–1464.
16. Klein M, Goncalves RB. Detection of *Tannerella forsythensis* and *Porphyromas gingivalis* by polymerase chain reaction in subjects with different periodontal staus. J Periodontol 2003: **74**: 798–802.
17. Lai C-H, Listgarten MA, Shirakawa M, Slots J. *Bacteroides forsythus* in adult gingivitis and periodontitis. Oral Microbiol Immunol 1987: **2**: 152–157.
18. Machtei EE, Hausmann E, Dunford R, Grossi S, Ho A, Chandler J, Zambon JJ, Genco RJ. Longitudinal study of predictive factors for periodontal disease and tooth loss. J Clin Periodontol 1999: **26**: 374–380.
19. Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. Periodontol 2000 1997: **14**: 216–248.
20. Papapanou PN, Neiderud AM, Papadimitriou A, Sandros J, Dahlen G. 'Checkerboard' assessments of periodontal microbiota and their antibody responses: a case control study. J Periodontol 2000: **68**: 651–656.
21. Poulter LW, Chilosi M, Seymour GJ, Hobbs S, Janossy G. Immunofluoresence membrane staining and cytochemistry, applied in combination for analyzing cell interactions *in situ*. In: Polak J, Noorden SV, eds. Immunocytochemistry: Practical Applications in Pathology and Biology. Bristol: Wright, 1983.
22. Sabet M, Lee SW, Nauman RK, Sims T, Ums HS. The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. Microbiology 2003: **149**: 3617–3627.
23. Seymour GJ, Gemmell E, Walsh LJ, Powell RN. Immunohistological analysis of experimental gingivitis in humans. Clin Exp Immunol 1988: **71**: 132–137.
24. Seymour GJ, Greenspan JS. The phenotypic characterization of lymphocytic subpopulations in established periodontal disease. J Periodontal Res 1979: **14**: 39–46.

25. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol 2000 2000: **20**: 82–121.
26. Socransky SS, Haffajee AD, Dzink JL. Relationship of subgingival microbial complexes to clinical features at sampled sites. J Clin Periodontol 1988: **15**: 440–444.
27. Takemoto T, Kurihara H, Dahlen G. Characterisation of *Bacteroides forsythus* isolates. J Clin Microbiol 1997: **35**: 1378–1381.
28. Trinchieri G. Cytokines acting on, or secreted by, macrophages during intracellular infection (IL-10, IL-12, IFN-γ). Curr Opin Immunol 1997: **9**: 17–23.
29. van Winkelhoff AG, Loos BG, van der Reijen WA, van der Velden U. *Porphyromonas gingivalis, Bacteroides forsythus* and other putative periodontal pathogens. J Clin Periodontol 2002: **29**: 1023–1028.
30. Yoneda M, Hirofuji T, Motooka N, Nozoe K, Shigenaga K, Anan H, et al. Humoral immune responses to S-layer-like proteins of *Bacteroides forsythus*. Clin Diagn Lab Immunol 2003: **10**: 383–387.
31. Yoneda M, Hirofuji Y, Anan H, Matasumoto A, Hamachi T, Nakayama K, et al. Mixed infection of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a murine abscess model: involvement of gingipains in a synergistic effect. J Periodontal Res 2001: **36**: 237–243.
32. Zambon JJ. Periodontal diseases: microbial factors. Ann Periodontol 1996: **1**: 879–925.