Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model

R.K-H. Orth, N.M. O'Brien-Simpson, S.G. Dashper and E.C. Reynolds

Oral Health CRC, Melbourne Dental School and Bio21 Institute, The University of Melbourne, Melbourne, Vic., Australia

Correspondence: Eric Reynolds, Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Melbourne, Vic. 3010 Australia Tel.: +613 9341 1547; fax: +61 3 9341 1596; E-mail: e.reynolds@unimelb.edu.au

Keywords: alveolar bone loss; animal model; chronic periodontitis; *Porphyromonas*; *Treponema*; virulence
Accepted 14 March 2011
DOI: 10.1111/j.2041-1014.2011.00612.x

SUMMARY

Chronic periodontitis is characterized by the destruction of the tissues supporting the teeth and has been associated with the presence of a subgingival polymicrobial biofilm containing *Porphyromonas gingivalis* and *Treponema denticola*. We have investigated the potential synergistic virulence of *P. gingivalis* and *T. denticola* using a murine experimental model of periodontitis. An inoculation regime of four intra-oral doses of 1×10^{10} *P. gingivalis* cells induced significant periodontal bone loss compared with loss in sham-inoculated mice, whereas doses of 1×10^{9} cells or lower did not induce bone loss. Inoculation with *T. denticola* with up to eight doses of 1×10^{10} cells failed to induce bone loss in this model. However, four doses of a co-inoculum of a 1 : 1 ratio of *P. gingivalis* and *T. denticola* at 5×10^{8} or 1×10^{9} total bacterial cells induced the same level of bone loss as four doses of 1×10^{10} *P. gingivalis* cells. Co-inoculation induced strong *P. gingivalis*-specific T-cell proliferative and interferon-γ-dominant cytokine responses, and induced a strong *T. denticola*-specific interferon-γ dominant cytokine response. Only at the higher co-inoculum dose of 1×10^{10} total cells was a *T. denticola*-specific T-cell proliferative response observed. These data show that *P. gingivalis* and *T. denticola* act synergistically to stimulate the host immune response and to induce alveolar bone loss in a murine experimental periodontitis model.

INTRODUCTION

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with specific oral bacteria and their products (Tanner, 1992; Garcia *et al.*, 1998; Tran & Rudney, 1999). It is characterized by the destruction of the tooth's supporting structures, including progressive alveolar bone loss that can lead ultimately to tooth loss (Lamont & Jenkinson, 1998; Tran *et al.*, 2000). Chronic periodontitis has been associated with three bacterial species in a subgingival plaque polymicrobial biofilm. The three bacterial species *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are proteolytic, anaerobic and together form a closely related aetiological cluster described as the 'Red complex' (Haffajee *et al.*, 1998; Socransky *et al.*, 1998; Tran & Rudney, 1999; Tran *et al.*, 2001). In a prospective clinical study, threshold levels of *P. gingivalis* and *T. denticola* at a periodontal site as measured by reverse transcription–polymerae chain reaction could predict clinical attachment loss at that site over the following 3 months, suggesting that *P. gingivalis* and *T. denticola* were important patho-

gens in the progression of chronic periodontitis (Byrne *et al.*, 2009).

Porphyromonas gingivalis is a gram-negative, anaerobic, asaccharolytic coccobacillus that is the most widely studied of the 'Red complex' bacteria and the most strongly associated with periodontal disease severity (Lamont & Jenkinson, 1998; Socransky *et al.*, 1998; O'Brien-Simpson *et al.*, 2003). *Treponema denticola* is an anaerobic spirochete. The difficulties in culturing oral spirochetes are believed to have led to the underestimation of the presence of *T. denticola* in chronic periodontitis (Canale-Parola, 1977; Salvador *et al.*, 1987; Umeda *et al.*, 1990; Chan *et al.*, 1993), which is now thought to play a significant role in the disease (Loesche, 1988; Chan & McLaughlin, 2000; Takeuchi *et al.*, 2001).

Various animal models of periodontitis have been used to study disease progression and host immune responses (Genco *et al.*, 1998). Subcutaneous abscess models have been used extensively to determine the virulence and pathogenicity of various bacteria associated with periodontitis including *P. gingivalis*, *T. denticola* and mixed infections (Feuille *et al.*, 1996; Kesavalu *et al.*, 1996, 1998; O'Brien-Simpson *et al.*, 2000a, 2005; Yoneda *et al.*, 2001; Washizu *et al.*, 2003; Frazer *et al.*, 2006). The relevance of these abscess models to periodontitis has been questioned and in recent years murine alveolar bone loss models were developed to test *P. gingivalis* virulence. These models have recently been adapted to test the virulence of *T. denticola* and polymicrobial infections (Kesavalu *et al.*, 2007; Lee *et al.*, 2009). These studies indicated that repeated intra-oral mono-inoculation with *T. denticola* cells resulted in colonization of the oral cavity of the animal, induction of a specific immune response and significant alveolar bone loss (Kesavalu *et al.*, 2007; Lee *et al.*, 2009). Furthermore, polymicrobial inoculation of *P. gingivalis*, *T. denticola* and *Tannerella forsythia* cells at a 1 : 1 : 1 ratio caused significantly higher levels of bone resorption than mono-inoculation with the individual species at the same level (Kesavalu *et al.*, 2007).

The aim of this current study was to investigate the effects of *T. denticola* and *P. gingivalis* both as individual and as mixed inoculations in the murine periodontitis model and to determine the immune responses to these infections.

METHODS

Bacterial strains and culture

Both *P. gingivalis* W50 and *T. denticola* ATCC 35405 were obtained from the Melbourne Dental School culture collection. An oral bacterial growth medium, adapted from NOS and GM-1 media (Leschine & Canale-Parola, 1980; Kesavalu *et al.*, 1997), which meets the growth requirements of both *P. gingivalis* and *T. denticola* was used as previously described (Veith *et al.*, 2009).

Porphyromonas gingivalis was grown in batch culture in a MK3 Anaerobic Workstation (Don Whitley Scientific Limited, Sydney, NSW, Australia) at 37°C with gas composition of 5% H_2 and 10% CO_2 in N_2 (BOC Gases Australia, Sydney, NSW, Australia) for 18–24 h to an optical density at 650 nm of 0.60 (O'Brien-Simpson *et al.*, 2000b, 2001). *Treponema denticola* was grown in continuous culture using a BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific, Edison, NJ) as previously described (Veith *et al.*, 2009). Briefly, the chemostat vessel volume was 900 ml and the bacterial culture was grown at 37°C with low agitation (50 r.p.m.) under a constant stream of anaerobic gas (5% CO_2 and 4% H_2 in N_2; BOC Gases Australia). The growth medium flow rate was 39.42 ml h^{-1} giving a dilution rate of 0.044 h^{-1} and a mean generation time of 15.75 h. Bacterial viability was determined by flow cytometry analysis using the BacLight™ LIVE/DEAD Bacterial Viability kit (Molecular Probes, Melbourne, Vic., Australia) as previously described (Orth *et al.*, 2009).

DNA analysis

DNA was extracted from 0.5 ml *P. gingivalis* culture containing $\sim 1.5 \times 10^9$ cells ml^{-1} or 1 ml *T. denticola* cell culture containing $\sim 7 \times 10^8$ cells ml^{-1} using a Qiagen DNeasy Tissue kit (Qiagen, Melbourne, Vic., Australia), with modifications to the manufacturer's protocol as previously described (Orth *et al.*, 2009). The extracted DNA was quantified with a Broad Range Quant-iT™ DNA Assay kit (Molecular Probes) following the standard protocol using a PerkinElmer Wallac Victor 3 1420 Multilable Counter (PerkinElmer, Melbourne, Vic., Australia). The number of bacterial cells was calculated as previously described for

T. denticola (Orth *et al.*, 2009) and for *P. gingivalis* by using a genomic size of 2,343,476 base pairs (Chen *et al.*, 2005).

Protein extraction

Bacterial protein extracts were produced using Triton X-114 as described previously and stored at −20°C until used (Pathirana *et al.*, 2006; Veith *et al.*, 2009).

Preparation of formalin-killed bacterial cells and immunization

Cultures of *T. denticola* were grown to the desired cell density and formalin-killed as previously described then stored at 4°C until use (Veith *et al.*, 2009). BALB/c mice were obtained from the animal facility at the Melbourne Dental School at The University of Melbourne, and animal experimentation was approved by the University of Melbourne animal ethics committee. For the T-cell proliferation and cytokine assays, formalin-killed *T. denticola* (1×10^{10} cells per mouse) were emulsified in adjuvant and used to immunize mice (50 μl per mouse). The first immunization was an intraperitoneal injection with complete Freund's adjuvant (Sigma-Aldrich, Sydney, NSW, Australia) and the second was a subcutaneous injection with incomplete Freund's adjuvant (Sigma-Aldrich) administered 30 days later.

Murine periodontitis model

The murine periodontitis model protocol was adapted from that previously described (O'Brien-Simpson *et al.*, 2005) and was approved by the University of Melbourne Ethics Committee for Animal Experimentation. *Porphyromonas gingivalis* strain W50 was used because it induces significant alveolar bone loss in this model and is strongly associated with human disease (O'Brien-Simpson *et al.*, 2005). Female BALB/c mice (12 per experimental group, 6–8 weeks old) were used, and the bacterial cell number per dose (25 μl) was determined by DNA quantification. The mice were sacrificed 8 weeks after the first oral inoculation (day 58) and maxillae were removed and processed as previously described (O'Brien-Simpson *et al.*, 2005). The area from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) on the buccal aspect of each molar tooth was determined using an Olympus D12 digital camera mounted on an Olympus SZ-CTV dissection microscope and measured using an OLYSIA BioReport soft imaging system (Olympus Australia Pty Ltd, Melbourne, Vic., Australia). Bone loss measurements were performed by an experienced examiner in a random and blinded protocol. Data are expressed as bacteria-induced bone loss (mm^2) i.e. the sum of the area from the ABC to the CEJ for each maxilla (mm^2) minus the sum of the measured bone loss area from the ABC to the CEJ for each maxilla (mm^2) of the control sham-inoculated group. For statistical analyses total bone loss areas were used.

To determine the number of doses of 1×10^{10} viable *P. gingivalis* cells were required to induce significant alveolar bone loss, mice received up to eight doses at intervals of 2 days. To determine the minimum number of bacterial cells required to induce bone loss, mice received either four doses of *P. gingivalis*, or eight doses of *T. denticola* of 1×10^{10}, 1×10^{9}, 1×10^{8}, or 1×10^{7} viable cells at intervals of 2 days. For mixed *T. denticola* and *P. gingivalis* inoculation, mice received four doses of a 1 : 1 ratio of viable *T. denticola* : *P. gingivalis* at 5×10^{8} or 1×10^{9} total cells per dose. A control group was sham inoculated in each experiment.

T-cell proliferation assay

T-cell proliferation was determined as previously described (Tam *et al.*, 2008). Submandibular lymph nodes (periodontitis model, 58 days after oral inoculation), inguinal, popliteal lymph nodes (T-cell stimulation study, 7 days after immunization) and spleens were removed and processed as previously described following sacrifice of the animals (Tam *et al.*, 2008). Lymph node (inguinal, popliteal and submandibular) T cells were isolated using $CD90^+$ beads and an AutoMACS cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions (O'Brien-Simpson *et al.*, 2005). Syngeneic spleen cells were prepared as a single cell suspension and the red blood cells were lysed, followed by gamma-irradiation (2200 rads) as previously described (Tam *et al.*, 2008). Briefly, 3×10^{5} T cells per well and 3×10^{5} syngeneic irradiated spleen cells per well were incubated in a 96-well

microtitre plate (Nunc/Thermo Fischer Scientific, Melbourne, Vic., Australia) with either *T. denticola* or *P. gingivalis* protein extracts in a final volume of 250 μl per well for 3.5 days at 37°C. Protein concentrations ranged from 0.005 to 25.0 μg ml^{-1}. For the final 18 h, 1 μCi [^{3}H]thymidine (GE Healthcare, Sydney, NSW, Australia) was added per well, after which cells were lysed and harvested onto glass-fibre filters using a Tomec Harvester96 Mach III cell Harvester (Tomec, New Haven, CT). The glass-fibre filters were dried and then treated with Betaplate Scint (PerkinElmer) and the amount of [^{3}H]thymidine was measured on a Wallac MicroBeta Trilix liquid scintillation counter (PerkinElmer).

Cytokine assay

ELISPOT cytokine assays were performed essentially as previously described, using 2 μg ml^{-1} of either *T. denticola* or *P. gingivalis* protein extract or 2 μg ml^{-1} formalin-killed *T. denticola* cells as the stimulating antigen (O'Brien-Simpson *et al.*, 2005). Briefly, ELISPOT plates (MultiScreen^{TM}-HA plates, Millipore, Sydney, NSW, Australia) were coated with 0.2 μg ml^{-1} of mouse cytokine capture antibodies specific for interleukin-4 (IL-4) and interferon-γ (IFN-γ) (eBiosciences, San Diego, CA). T cells were extracted from the submandibular lymph nodes (periodontitis model, 58 days after oral inoculation) or from the inguinal and popliteal lymph nodes (T-cell stimulation study, 7 days after immunization) and spleens (from naive mice) were removed as a source of syngeneic antigen-presenting cells and processed as previously described (Tam *et al.*, 2008). Following washing and blocking steps, plates with 3×10^5 T cells, 3×10^5 spleen cells and antigen in a final volume of 150 μl per well were incubated at 37°C for 48 h. Plates were then washed and incubated for 2 h with 2 μg ml^{-1} of cytokine-specific biotinylated conjugated antibodies (eBiosciences) specific for IL-4, IFN-γ, IL-10, IL-5, IL-2, tumour necrosis factor-α and granulocyte–macrophage colony-stimulating factor (GM-CSF) in phosphate-buffered saline (PBS, 15 mM NaCl, 0.3 mM KCl, 1 mM Na_2HPO_4, 0.15 mM KH_2PO_4, pH 7.4)/Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) (1 : 1, volume/volume). Plates were further washed and incubated for 1 h with 1 : 1000 streptavidin–alkaline phosphatase conjugate (Roche, Sydney, NSW, Australia) in PBS/DMEM, then washed six times, and developed using the substrate SIGMA-*FAST*^{TM} BCIP®/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; Sigma-Aldrich) as per the manufacturer's instructions. The plates were then incubated at room temperature for visualization of spots (typically ~20 min), and the reaction was stopped by washing the plates in water. The spots were counted using ELISPOT READER LITE (version 2.9.; Autoimmun Diagnostika, Straßberg, Germany) and data are expressed as spot-forming cells per million T cells.

Statistical analysis

The bone loss data were statistically analysed using one-way analysis of variance and Dunnett's T3 test (Norusis, 1993). The ELISPOT data were found to be not normally distributed using Levene's test for homogeneity of variances, so were statistically analysed using the Mann–Whitney *U* Wilcoxon rank sum test.

RESULTS

Analysis of alveolar bone loss induced by *P. gingivalis* and *T. denticola* in the murine periodontitis model

To determine the number of doses of 1×10^{10} *P. gingivalis* cells that was required to induce a significant difference in bone loss compared with the control sham-inoculated group, groups of 12 mice received eight, four, two or one dose of 1×10^{10} *P. gingivalis* cells. Mice that received four and eight doses of 1×10^{10} *P. gingivalis* cells had significantly ($P < 0.05$) greater alveolar bone loss compared with the sham-inoculated group (Fig. 1). Further, mice which received the eight doses of 1×10^{10} *P. gingivalis* cells had significantly ($P < 0.05$) greater alveolar bone loss compared with all of the groups (Fig. 1). No significant difference in bone loss was observed in mice that received two or one dose of 1×10^{10} *P. gingivalis* cells compared with the sham-inoculated group.

To determine the number of *P. gingivalis* cells that was required to induce a significant difference in bone loss compared with the control sham-inoculated group, groups of 12 mice received four doses of

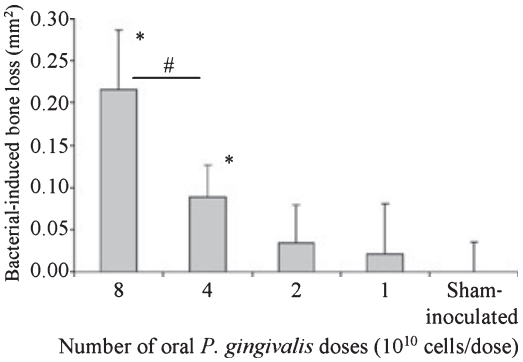

Figure 1 Dose-dependent *Porphyromonas gingivalis* induced alveolar bone loss. BALB/c mice were orally inoculated with eight, four, two or one doses of 1×10^{10} *P. gingivalis* cells. *Significantly different from the sham-inoculated group ($P < 0.05$). #Significantly different ($P < 0.05$). Data are expressed as bacterially induced bone loss (mm^2), i.e. the sum of the measured bone loss area from the alveolar bone crest (ABC) to the cemento–enamel junction (CEJ) for each maxilla tooth (mm^2) minus the sum of the measured bone loss area from the ABC to the CEJ for each maxilla tooth (mm^2) of the control sham-inoculated group. Error bars represent one standard deviation.

1×10^{10}, 1×10^{9}, 1×10^{8} or 1×10^{7} *P. gingivalis* cells. Analysis of the bone loss induced in each group found that only mice that received four doses of 1×10^{10} *P. gingivalis* cells had significantly ($P < 0.05$) greater bone loss compared with the control sham-inoculated (data not shown). Similar experiments with *T. denticola* to determine the number of doses and number of cells required to induce a significant difference in bone loss compared with the control sham-inoculated group, found that under these conditions *T. denticola* alone did not induce a significant difference in bone loss compared with the control group (data not shown).

To determine whether *P. gingivalis* and *T. denticola* act synergistically to enhance virulence in the murine periodontitis model, mice received four doses of a co-inoculum of a 1 : 1 ratio of *T. denticola* : *P. gingivalis* at 5×10^{8} or 1×10^{9} total cells per dose. These data were compared with those for control sham-inoculated mice, mice that received four doses of 1×10^{10} or 1×10^{9} *P. gingivalis* cells and mice that received eight doses of 1×10^{10} or 1×10^{9} *T. denticola* cells (Fig. 2). Inoculation of mice with eight doses of 1×10^{10} or 1×10^{9} *T. denticola* cells or four doses of 1×10^{9} *P. gingivalis* cells did not induce significantly greater bone loss compared with the sham-inoculated control group (Fig. 2). However, mice that received four doses of a co-inoculum of *T. denticola* : *P. gingivalis* at 5×10^{8} or 1×10^{9} total cells had significantly ($P < 0.05$) greater bone loss compared with the sham-inoculated group (Fig. 2). Further, the amount of bone loss induced in the

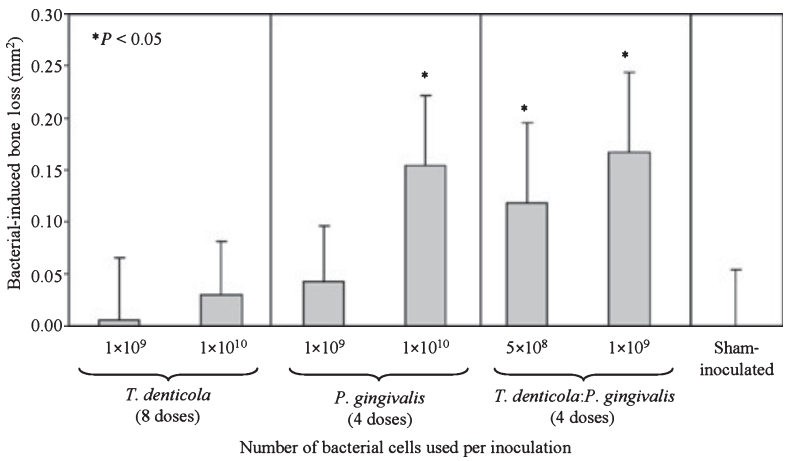

Figure 2 Alveolar bone loss following oral inoculation with *Treponema denticola*, *Porphyromonas gingivalis*, or a mixed *T. denticola*/*P. gingivalis* inoculum. BALB/c mice were orally inoculated with either eight doses of *T. denticola* at 1×10^{9} or 1×10^{10} cells per dose, four doses of *P. gingivalis* at 1×10^{9} or 1×10^{10} cells per dose, or four doses of a 1 : 1 ratio of *T. denticola* : *P. gingivalis* at 5×10^{8} or 1×10^{9} total cells per dose. *Significantly different from sham-inoculated group ($P < 0.05$). Data are expressed as bacterially induced bone loss (mm^2), i.e. the sum of the measured bone loss area from the alveolar bone crest (ABC) to the cemento–enamel junction (CEJ) for each maxilla tooth (mm^2) minus the sum of the measured bone loss area from the ABC to the CEJ for each maxilla tooth (mm^2) of the control sham-inoculated group. Error bars represent one standard deviation.

T. denticola : *P. gingivalis* co-inoculum groups (four doses of 5×10^8 or 1×10^9 total cells) was similar to the bone loss induced in mice that received four doses of 1×10^{10} *P. gingivalis* cells.

P. gingivalis and *T. denticola* antigen-specific proliferative responses from T cells isolated from mice in the experimental periodontitis model

Submandibular T cells were isolated from each of the inoculated and sham-inoculated groups in the murine periodontitis model and stimulated with up to 25.0 μg ml^{-1} of a protein extract from *T. denticola* and *P. gingivalis*. T cells from mice that received eight doses of 1×10^9 *T. denticola* or four doses of 1×10^9 *P. gingivalis* did not respond to either *T. denticola* or *P. gingivalis* protein extracts (data not shown). T cells from mice that were inoculated with eight doses of 1×10^{10} *T. denticola* were not stimulated by incubation with *T. denticola* protein extract at concentrations up to 5.0 μg ml^{-1}. These T cells appeared to exhibit a low level of proliferation when incubated with a relatively high concentration of *P. gingivalis* protein extract (0.156 μg ml^{-1}), although this was not statistically significant (Fig. 3).

T cells from mice that were inoculated with four doses of 1×10^{10} *P. gingivalis* cells were highly stimulated when incubated with *P. gingivalis* protein extract and exhibited the highest proliferative response (maximal proliferation), at an antigen concentration of 0.005 μg per well. These T cells also displayed a low level of stimulation in response to a relatively high concentration of a *T. denticola* protein extract (0.156 μg ml^{-1}), although this was not statistically significant (Fig. 3).

Incubation of T cells from mice that were inoculated with four doses of a 1 : 1 ratio of *T. denticola* : *P. gingivalis* at 1×10^9 total cells per dose with *T. denticola* protein extract induced a weak but significant ($P < 0.05$) proliferative response and exhibited maximum proliferation at an antigen concentration of 0.01 μg ml^{-1}. These T cells were highly stimulated when incubated with *P. gingivalis* protein extract, with a maximum proliferative response at an antigen concentration of 0.078 μg ml^{-1} (Fig. 3). T cells from mice that were inoculated with four doses of a 1 : 1 ratio of *T. denticola* : *P. gingivalis* at 5×10^8 total cells per dose only had a significant ($P < 0.05$) proliferative response when incubated with *P. gingivalis* protein extract, with a maximal proliferative response at an antigen concentration of 0.01 μg ml^{-1} (Fig. 3).

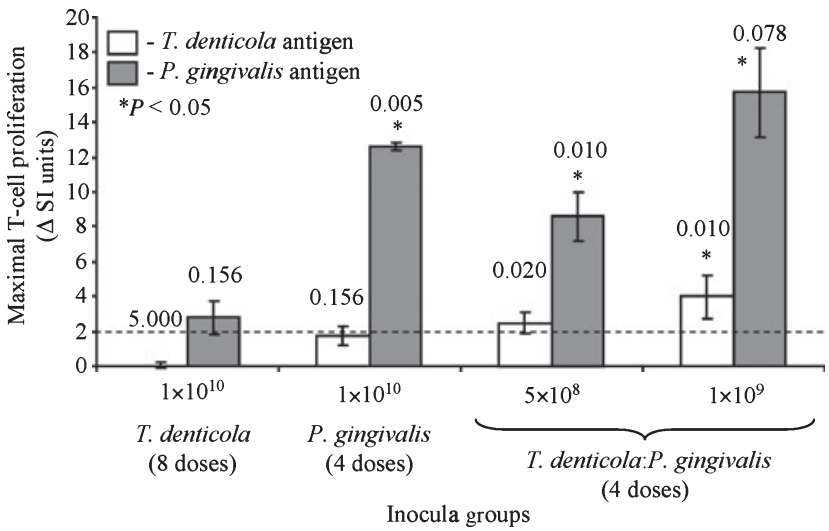

Figure 3 T-cell proliferation responses specific to *Treponema denticola* and *Porphyromonas gingivalis* antigens. Maximal T cell proliferation stimulated by *T. denticola* or *P. gingivalis* protein extract antigens. T cells were taken from BALB/c mice orally inoculated with eight doses of 1×10^{10} *T. denticola* cells, four doses of 1×10^{10} *P. gingivalis* cells, or four doses of 5×10^8 and 1×10^9 cells of a 1 : 1 ratio of *T. denticola* : *P. gingivalis*. The amounts of stimulatory protein (μg ml^{-1}) required for the maximal T-cell proliferation are shown above each bar. T-cell proliferation data are expressed as stimulatory index (SI), where SI is the counts per minute divided by the negative control (no antigen) counts per minute. Error bars represent one standard deviation. Data analysed using Student's *t*-test with the comparative mean set at 2.00 SI units.

T-cell cytokine response to *P. gingivalis* and *T. denticola* protein extracts from T cells isolated from mice in the periodontitis model

Submandibular T cells were isolated from each of the inoculated and sham-inoculated groups in the mouse periodontitis model, stimulated with a protein extract from *T. denticola* and *P. gingivalis* and the number of IL-4 and IFN-γ secreting T cells was determined by ELISPOT (Fig. 4). T cells from mice that received eight doses of 1×10^9 *T. denticola* or four doses of 1×10^9 *P. gingivalis* were not stimulated by either *T. denticola* or *P. gingivalis* protein extracts (data not shown). All bacteria-inoculated groups incubated with *T. denticola* protein extract had a significantly ($P < 0.05$) higher number of IFN-γ-secreting T cells compared with the sham-inoculated group. Furthermore, IFN-γ-secreting T cells were the predominant ($P < 0.05$) T cells in response to *T. denticola* protein extract in T cells extracted from mice inoculated with eight doses of 1×10^{10} *T. denticola* cells, four doses of a co-inoculum of *T. denticola* : *P. gingivalis* at 5×10^8 or 1×10^9 total cells (Fig. 4A). In contrast, mice inoculated with four doses of 1×10^{10} *P. gingivalis* cells had a significant ($P < 0.05$) number of IL-4-secreting T cells in response *T. denticola* protein extract, which was similar to the number of IFN-γ-secreting T cells (Fig. 4A). Mice that had received four doses of 1×10^{10} *P. gingivalis* cells or four doses of a co-inoculum of *T. denticola* : *P. gingivalis* at 5×10^8 or 1×10^9 total cells had a predominant and significantly ($P < 0.05$) higher number of IFN-γ-secreting T cells in response to *P. gingivalis* protein extract compared with the sham-inoculated controls (Fig. 4B). T cells from mice inoculated with eight doses of 1×10^{10} *T. denticola* cells had no significant IL-4 or IFN-γ response when stimulated with *P. gingivalis* protein extract (Fig. 4B). Only mice that received four doses of 5×10^8 of a 1 : 1 ratio of *T. denticola* : *P. gingivalis* had a significant IL-4 response to *P. gingivalis* protein extract (Fig. 4B).

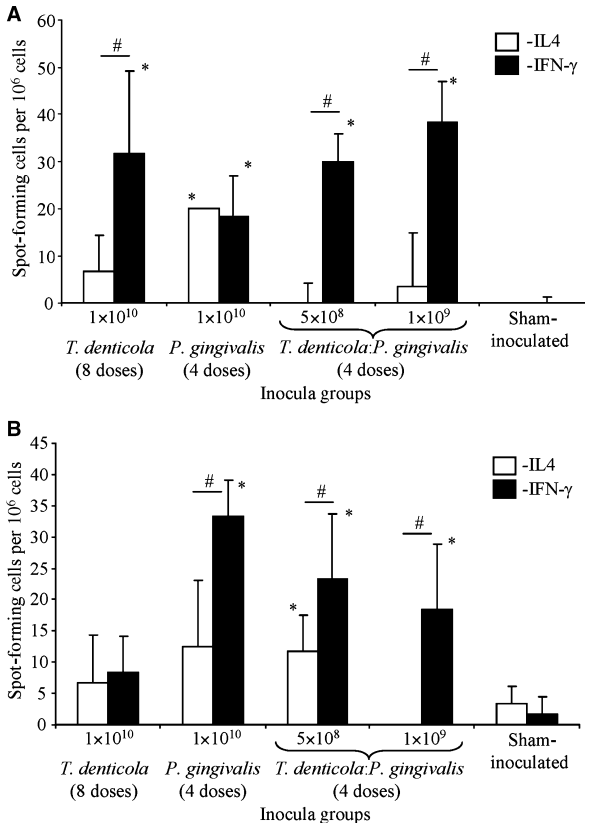

Figure 4 Cytokine responses of mice inoculated with *Treponema denticola* and/or *Porphyromonas gingivalis*. Interleukin-4 (IL-4) and interferon-γ (IFN-γ) cytokine responses to 2 μg ml^{-1} of a *T. denticola* (A) or *P. gingivalis* (B) protein extract from BALB/c mice orally inoculated with either eight doses of 1×10^{10} *T. denticola*, four doses of 1×10^{10} *P. gingivalis*, or four doses of 5×10^8 or 1×10^9 of a 1 : 1 ratio of *T. denticola* : *P. gingivalis*. Error bars represent one standard deviation. *Significantly different ($P \leq 0.05$) from the sham-inoculated group. #Significant difference between cytokine responses ($P \leq 0.05$).

T-cell proliferation and cytokine responses to *T. denticola* antigens

Popliteal and inguinal lymph node T cells were isolated 7 days after mice were immunized with 1×10^{10} formalin-killed *T. denticola* whole cells and stimulated *in vitro* with *T. denticola* protein extract or formalin-killed *T. denticola* whole cells to determine the *T. denticola*-specific T-cell proliferative and cytokine responses (Fig. 5). The *T. denticola* protein extract stimulated a strong T-cell proliferative response that was significantly higher ($P < 0.05$) than control at 0.1 μg ml^{-1}. Furthermore, formalin-killed *T. denticola* whole cells stimulated cytokine secretion with IL-10, IL-4, IFN-γ, GM-CSF and IL-2 all being significantly ($P < 0.05$) higher than in the control, with the order of secretion being IL-10 >> IL-4 > IFN-γ = GM-CSF > IL-2.

DISCUSSION

Experimental animal models of bacterially induced periodontitis (alveolar bone loss models) are invalu-

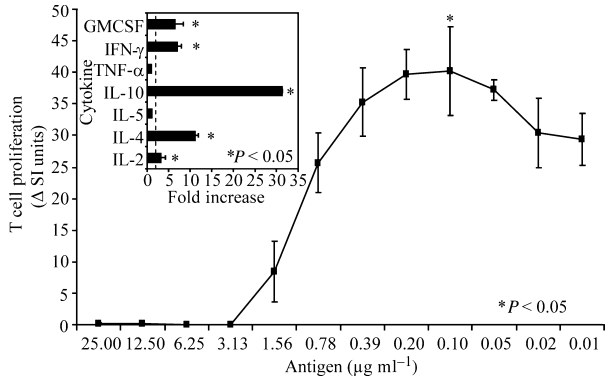

Figure 5 T-cell proliferation and cytokine response to *Treponema denticola* whole cells. T cells were isolated from popliteal and inguinal lymph nodes of BALB/c mice 7 days after the second immunization with 1×10^{10} *T. denticola* formalin-killed whole cells and incubated with *T. denticola* protein extract. T-cell proliferation data are expressed as stimulatory index (SI), where SI is the counts per minute divided by the negative control (no antigen) counts per minute. Data were analysed using Student's *t*-test with the comparative mean set at 2.00 SI units. Insert shows cytokine responses to 2 μg ml^{-1} of *T. denticola* formalin-killed whole cells, data expressed as fold increase (i.e. spot-forming cells of test antigen/spot-forming cells of the no antigen control). Error bars represent one standard deviation. *Significant difference between cytokine responses ($P \leq 0.05$). Data analysed using Student's *t*-test with the comparative mean set at 2.00-fold increase.

able to the study of disease progression, determination of bacterial virulence factors and host immune responses (Klausen *et al.*, 1991; Baker *et al.*, 1994; Genco *et al.*, 1998). The majority of studies using murine alveolar bone loss models to date have focused on mono-infection with a single bacterial species, however, as periodontitis is a polymicrobial disease studying a single species in the model may give little insight into the virulence of that species when together with other periodontal pathogens (Baker *et al.*, 1994; Zubery *et al.*, 1998; Kesavalu *et al.*, 1999, 2007; Washizu *et al.*, 2003; O'Brien-Simpson *et al.*, 2005; Lee *et al.*, 2009).

Repeated inoculation with *P. gingivalis* has been shown to induce alveolar bone loss in the mouse (Evans *et al.*, 1992; Baker *et al.*, 1994; Rajapakse *et al.*, 2002; Gonzalez *et al.*, 2003; O'Brien-Simpson *et al.*, 2005; Kesavalu *et al.*, 2007). More recently, inoculation with *T. denticola* has been shown to induce alveolar bone loss in experimental periodontitis models (Kesavalu *et al.*, 2007; Lee *et al.*, 2009). In addition, rats that were repeatedly inoculated with a mixture of *T. denticola*, *P. gingivalis* and *T. forsythia* cells were shown to produce significantly more bone loss than animals inoculated with only the individual species (Kesavalu *et al.*, 2007).

In the current study we attempted to determine the minimum number of intra-oral inoculations and the least number of cells per inoculation of *P. gingivalis* and *T. denticola* necessary to induce alveolar bone loss in mice in an approach aimed at studying the synergistic virulence of these species (Figs 1 and 2). Interestingly, intra-oral inoculation with *T. denticola* did not induce significant alveolar bone loss in this model at up to eight inoculations of 1×10^{10} cells (Fig. 2). These results contrast with previous studies that reported that inoculation with *T. denticola* induced alveolar bone loss in the rat and mouse (Kesavalu *et al.*, 2007; Lee *et al.*, 2009). This may be attributed to differences in the models, such as choice of species (rats or mice (Kesavalu *et al.*, 2007; Lee *et al.*, 2009)), mouse strain, number of inoculations and other experimental protocol differences. Lee *et al.* (2009) used seven doses of 1×10^{9} *T. denticola* cells to achieve significant bone loss; however, in the current study using the same *T. denticola* strain we were unable to observe significant levels of bone loss with eight doses of 1×10^{10} *T. denticola* cells. The inconsistencies between the Lee *et al.* (2009) study and ours may be a reflection of the different mouse strains used as Baker (2005) showed that there were different levels of susceptibility to bone loss induced by *P. gingivalis* inoculation in BALB/c and C57BL/6 mice. These mouse strain differences may also apply to the bone loss caused by *T. denticola.* In addition Lee *et al.* (2009) used a longer period between the last *T. denticola* inoculation and killing of the animals than in our study.

Recently, a rat model of disease has been used to examine the interactions of *P. gingivalis* with *T. denticola* and *Tannerella forsythia* (Verma *et al.*, 2010a,b). These authors reported that there was no synergy between *P. gingivalis* and *T. denticola* in producing bone loss in their model. However, they demonstrated that an inoculum of a 1 : 1 ratio of *P. gingivalis* and *T. denticola* produced significantly more vertical alveolar bone loss than mono-infection using the same total number of *P. gingivalis* or *T. denticola* cells (Verma *et al.*, 2010b). They used a different approach to determine synergy and did not investigate the minimum inoculation regimen needed to cause significant alveolar bone loss. By determining

the minimum *P. gingivalis* inoculation regimen needed to cause significant alveolar bone loss we were able to demonstrate that the addition of *T. denticola* to the *P. gingivalis* inoculum caused an enhanced virulence. In fact, significant alveolar bone loss occurred with a 20-fold lower number of total bacterial cells and 40-fold fewer *P. gingivalis* cells (Fig. 2). Further, the *P. gingivalis* and *T. denticola* co-inoculum of 5×10^8 total cells contained four-fold fewer *P. gingivalis* cells than the 1×10^9 *P. gingivalis* cell inoculum, which did not induce bone loss, yet this co-inoculum induced the same level of bone loss as the 1×10^{10} *P. gingivalis* cell inoculum. This synergy may relate to metabolic co-operativity between the species or an altered immune response to the mixed infection.

An investigation of the immune response to these inocula showed that there was little or no T-cell proliferation when T cells from mice that were inoculated with *T. denticola*, *P. gingivalis* or the co-inoculum (5×10^8 total cells) were incubated with *T. denticola* protein extract; although a significant *T. denticola* T-cell proliferative response was observed in T cells from mice that had received the higher (1×10^9 total cells) *T. denticola*/*P. gingivalis* co-inoculum (Fig. 3). The dose of *T. denticola* in the co-inoculum that produced a significant T-cell response was 20-fold less than the *T. denticola* monospecies inoculum that produced no T-cell response. There was a robust proliferative response when T cells from mice inoculated with *P. gingivalis* (four doses of 1×10^{10} cells) or the *P. gingivalis*/*T. denticola* co-inoculum (four doses of either 5×10^8 or 1×10^9 cells) were incubated with the *P. gingivalis* protein extract. These results also suggest a synergistic response because a 20-fold lower *P. gingivalis* cell number in the co-inoculum produced a slightly higher response than the single inoculum cell dose, albeit the maximum response was at a slightly higher *in vitro* antigen concentration (Fig. 3).

All mice inoculated with *P. gingivalis* that developed bone loss, whether as part of a single or mixed inoculation, had a significant and predominately higher IFN-γ response when stimulated by the *P. gingivalis* protein extract (Fig. 4), indicating an inflammatory, T helper type 1 (Th1) -biased response. Similarly, when stimulated by the *T. denticola* protein extract, there was a significant and predominant higher IFN-γ response in all mice that were inoculated with *T. denticola*, either as a single inoculation or as a co-inoculum (Fig. 4). Again, the response was higher for the 20-fold lower *T. denticola* cell number in the co-inoculum. Surprisingly, although mono-inoculation with *T. denticola* at eight doses of 1×10^{10} cells did not induce significant alveolar bone loss or a T-cell proliferative response, it did induce *T. denticola*-specific IFN-γ-secreting T cells (Fig. 4). This suggests that *T. denticola* at this inoculum dose did infect mice, therefore producing a T-cell response. This immune phenotype of a very low T-cell proliferative response but an antigen-specific IFN-γ T-cell response is typical of a *Treponema pallidum* infection, where at certain points in the syphilis disease cycle there are high and very low *T. pallidum*-specific T-cell proliferative responses (Arroll *et al.*, 1999). In a separate experiment *T. denticola* antigens induced a strong T-cell proliferative (40.2 ± 7.0 ΔSI units) response with a response maximum at 0.1 μg ml^{-1} in *T. denticola* whole cell immunized mice (Fig. 5). Furthermore, these *T. denticola* proliferative T cells had a predominant IL-10 followed by IL-4 > IFN-γ = GM-CSF > IL-2 cytokine response. This cytokine profile is typical of certain points in the *T. pallidum* disease cycle where the Th1 cell proliferative response is low and the T-cell cytokine response is IL-10 dominant (Arroll *et al.*, 1999; Podwinska *et al.*, 2000). Taken together, these data indicate that *T. denticola* does induce a T-cell response after intra-oral inoculation and that it may induce cycles of high and low T-cell proliferation in a similar way to *T. pallidum*. Furthermore, when *P. gingivalis* is present with *T. denticola* the response is greater both in T-cell proliferation and in IFN-γ secretion. The predominant T-cell cytokine response to either *T. denticola* or *P. gingivalis* was IFN-γ, suggesting a bias towards an inflammatory Th1 response, which is associated with loss of alveolar bone (O'Brien-Simpson *et al.*, 2000a, 2005; Stashenko *et al.*, 2007). The IL-4 and IFN-γ cytokine profiles in response to periodontal bacterial antigens have been used to determine the Th1/Th2 subset response in the mouse periodontitis model (Choi *et al.*, 2001; O'Brien-Simpson *et al.*, 2005; Tam *et al.*, 2008). When *P. gingivalis* has been used in the mouse periodontitis model, alveolar bone loss has been associated with an increase in T-cell-derived IFN-γ (Baker *et al.*, 1999; O'Brien-Simpson *et al.*, 2005). Furthermore, IFN-γ has been shown to increase in the diseased tissues of periodontitis patients (Lundqvist *et al.*, 1994; Takeichi

et al., 1998) and a lack of IL-4 in inflamed gingival tissue has been associated with the progression of disease (Shapira *et al.*, 1992). Our data show that a co-infection with *P. gingivalis* and *T. denticola* in the mouse model demonstrated synergy with respect to secretion of a Th1 cytokine profile and alveolar bone loss.

The continuing development of this model will allow the assessment of the virulence determinants of *T. denticola* and *P. gingivalis* that promote synergism in the stimulation of the host immune response and development of alveolar bone loss.

ACKNOWLEDGEMENTS

We wish to thank Jenny Davis for caring for the mice used in the periodontitis model. This work was supported by NHMRC grant 251708.

REFERENCES

Arroll, T.W., Centurion-Lara, A., Lukehart, S.A. and Van Voorhis, W.C. (1999) T-cell responses to *Treponema pallidum* subsp. *pallidum* antigens during the course of experimental syphilis infection. *Infect Immun* **67**: 4757–4763.

Baker, P.J. (2005) Genetic control of the immune response in pathogenesis. *J Periodontol* **76**: 2042–2046.

Baker, P.J., Evans, R.T. and Roopenian, D.C. (1994) Oral infection with *Porphyromonas gingivalis* induces alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol* **39**: 1035–1040.

Baker, P.J., Dixon, M., Evans, R.T., Dufour, L., Johnson, E. and Roopenian, D.C. (1999) $CD4^+$ T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. *Infect Immun* **67**: 2804–2809.

Byrne, S.J., Dashper, S.G., Darby, I.B., Adams, G.G., Hoffmann, B. and Reynolds, E.C. (2009) Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol Immunol* **24**: 469–477.

Canale-Parola, E. (1977) Physiology and evolution of spirochetes. *Bacteriol Rev* **41**: 181–204.

Chan, E.C. and McLaughlin, R. (2000) Taxonomy and virulence of oral spirochetes. *Oral Microbiol Immunol* **15**: 1–9.

Chan, E.C., Siboo, R., Touyz, L.Z., Qui, Y.S. and Klitorinos, A. (1993) A successful method for quantifying viable oral anaerobic spirochetes. *Oral Microbiol Immunol* **8**: 80–83.

Chen, T., Abbey, K., Deng, W.J. and Cheng, M.C. (2005) The bioinformatics resource for oral pathogens. *Nucleic Acids Res* **33** (Web Server issue): W734–W740.

Choi, J., Borrello, M.A., Smith, E., Cutler, C.W., Sojar, H. and Zauderer, M. (2001) Prior exposure of mice to *Fusobacterium nucleatum* modulates host response to *Porphyromonas gingivalis*. *Oral Microbiol Immunol* **16**: 338–344.

Evans, R.T., Klausen, B., Sojar, H.T. *et al.* (1992) Immunization with *Porphyromonas* (*Bacteroides*) *gingivalis* fimbriae protects against periodontal destruction. *Infect Immun* **60**: 2926–2935.

Feuille, F., Ebersole, J.L., Kesavalu, L., Stepfen, M.J. and Holt, S.C. (1996) Mixed infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a murine lesion model: potential synergistic effects on virulence. *Infect Immun* **64**: 2094–2100.

Frazer, L.T., O'Brien-Simpson, N.M., Slakeski, N. *et al.* (2006) Vaccination with recombinant adhesins from the RgpA-Kgp proteinase-adhesin complex protects against *Porphyromonas gingivalis* infection. *Vaccine* **24**: 6542–6554.

Garcia, L., Tercero, J.C., Legido, B., Ramos, J.A., Alemany, J. and Sanz, M. (1998) Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromona gingivalis* by multiplex PCR. *J Periodontal Res* **33**: 59–64.

Genco, C.A., Van Dyke, T. and Amar, S. (1998) Animal models for *Porphyromonas gingivalis*-mediated periodontal disease. *Trends Microbiol* **6**: 444–449.

Gonzalez, D., Tzinabos, A.O., Genco, C.A. and Gibson, F.C. 3rd (2003) Immunization with *Porphyromonas gingivalis* capsular polysaccharide prevents *P. gingivalis*-elicited oral bone loss in a murine model. *Infect Immun* **71**: 2283–2287.

Haffajee, A.D., Cugini, M.A., Tanner, A. *et al.* (1998) Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. *J Clin Periodontol* **25**: 346–353.

Kesavalu, L., Holt, S.C. and Ebersole, J.L. (1996) Trypsin-like protease activity of *Porphyromonas gingivalis* as a potential virulence factor in a murine lesion model. *Microb Pathog* **20**: 1–10.

Kesavalu, L., Walker, S.G., Holt, S.C., Crawley, R.R. and Ebersole, J.L. (1997) Virulence characteristics of oral treponemes in a murine model. *Infect Immun* **65**: 5096–5102.

Kesavalu, L., Holt, S.C. and Ebersole, J.L. (1998) Virulence of a polymicrobic complex, *Treponema denticola* and *Porphyromonas gingivalis*, in a murine model. *Oral Microbiol Immunol* **13**: 373–377.

Kesavalu, L., Holt, S.C. and Ebersole, J.L. (1999) Lack of humoral immune protection against *Treponema denticola* virulence in a murine model. *Infect Immun* **67**: 5736–5746.

Kesavalu, L., Sathishkumar, S., Bakthavatchalu, V. *et al.* (2007) Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. *Infect Immun* **75**: 1704–1712.

Klausen, B., Evans, R.T., Ramamurthy, N.S. *et al.* (1991) Periodontal bone level and gingival proteinase activity on gnobiotic rats immunized with *Bacteroides gingivalis*. *Oral Microbiol Immunol* **6**: 193–201.

Lamont, R.J. and Jenkinson, H.F. (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* **62**: 1244–1263.

Lee, S.F., Andrian, E., Rowland, E. and Marquez, I.C. (2009) Immune response and alveolar bone resorption in a mouse model of *Treponema denticola* infection. *Infect Immun* **77**: 694–698.

Leschine, S.B. and Canale-Parola, E. (1980) Rifampin as a selective agent for isolation of oral spirochetes. *J Clin Microbiol* **12**: 792–795.

Loesche, W.J. (1988) The role of spirochetes in periodontal disease. *Adv Dent Res* **2**: 275–283.

Lundqvist, C., Baranov, V., Teglund, S., Hammarstrom, S. and Hammarstrom, M. (1994) Cytokine profile and ultrastructure of intraepithelial gd T cells in chronically inflamed human gingiva suggest a cytotoxis effector function. *J Immunol* **153**: 2302–2312.

Norusis, M.J. (1993) SPSS for Windows: Base systems user's guide. SPSS Inc.: Chicago, IL, USA.

O'Brien-Simpson, N.M., Black, C.L., Bhogal, P.S. *et al.* (2000a) Serum immunoglobulin G (IgG) and IgG subclass responses to the RgpA-Kgp proteinase-adhesin complex of *Porphyromonas gingivalis* in adult periodontitis. *Infect Immun* **68**: 2704–2712.

O'Brien-Simpson, N.M., Paolini, R.A. and Reynolds, E.C. (2000b) RgpA-Kgp peptide-based immunogens provide protection against *Porphyromonas gingivalis* challenge in a murine lesion model. *Infect Immun* **68**: 4055–4063.

O'Brien-Simpson, N.M., Paolini, R.A., Hoffmann, B., Slakeski, N., Dashper, S.G. and Reynolds, E.C. (2001) Role of RgpA, RgpB, and Kgp proteinases in virulence of *Porphyromonas gingivalis* W50 in a murine lesion model. *Infect Immun* **69**: 7527–7534.

O'Brien-Simpson, N.M., Veith, P.D., Dashper, S. and Reynolds, E. (2003) *Porphyromonas gingivalis* gingipains: the molecular teeth of a microbial vampire. *Curr Protein Pept Sci* **4**: 409–426.

O'Brien-Simpson, N.M., Pathirana, R.D., Paolini, R.A. *et al.* (2005) An immune response directed to proteinase and adhesin functional epitopes protects against *Porphyromonas gingivalis*-induced periodontal bone loss. *J Immunol* **175**: 3980–3989.

Orth, R.K.H., O'Brien-Simpson, N.M., Dashper, S.G., Walsh, K.A. and Reynolds, E.C. (2009) An efficient method for enumerating oral spirochetes using flow cytometry. *J Microbiol Methods* **80**: 123–128.

Pathirana, R.D., O'Brien-Simpson, N.M., Veith, P.D., Riley, P.F. and Reynolds, E.C. (2006) Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. *Microbiology* **152**: 2381–2394.

Podwinska, J., Lusiak, M., Zaba, R. and Bowszyc, J. (2000) The pattern and level of cytokines secreted by Th1 and Th2 lymphocytes of syphilitic patients correlate to the progression of the disease. *FEMS Immunol Med Microbiol* **28**: 1–14.

Rajapakse, P.S., O'Brien-Simpson, N.M., Slakeski, N., Hoffmann, B. and Reynolds, E.C. (2002) Immunization with the RgpA-Kgp proteinase-adhesin complexes of *Porphyromonas gingivalis* protects against periodontal bone loss in the rat periodontitis model. *Infect Immun* **70**: 2480–2486.

Salvador, S.L., Syed, S.A. and Loesche, W.J. (1987) Comparison of three dispersion procedures for quantitative recovery of cultivable species of subgingival spirochetes. *J Clin Microbiol* **25**: 2230–2232.

Shapira, L., Van Dyke, T.E. and Hart, T.C. (1992) A localized absence of interleukin-4 triggers periodontal disease activity: a novel hypothesis. *Med Hypotheses* **39**: 319–322.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. and Kent, R.L. Jr (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**: 134–144.

Stashenko, P., Goncalves, R.B., Lipkin, B., Ficarelli, A., Sasaki, H. and Campos-Neto, A. (2007) Th1 immune response promotes severe bone resorption caused by *Porphyromonas gingivalis*. *Am J Pathol* **170**: 203–213.

Takeichi, O., Saito, I., Okamoto, Y., Tsurumachi, T. and Saito, T. (1998) Cytokine regulation on the synthesis of nitric oxide *in vivo* by chronically infected human polymorphonuclear leucocytes. *Immunology* **93**: 275–280.

Takeuchi, Y., Umeda, M., Sakamoto, M., Benno, Y., Huang, Y. and Ishikawa, I. (2001) *Treponema socranskii*, *Treponema denticola*, and *Porphyromonas*

gingivalis are associated with severity of periodontal tissue destruction. *J Periodontol* **72**: 1354–1363.

Tam, V., O'Brien-Simpson, N.M., Pathirana, R.D., Frazer, L.T. and Reynolds, E.C. (2008) Characterization of T cell responses to the RgpA-Kgp proteinase-adhesin complexes of *Porphyromonas gingivalis* in BALB/c mice. *J Immunol* **181**: 4150–4158.

Tanner, A. (1992) Microbial etiology of periodontal diseases. Where are we? Where are we going? *Curr Opin Dent* **2**: 12–24.

Tran, S.D. and Rudney, J.D. (1999) Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. *J Clin Microbiol* **37**: 3504–3508.

Tran, S.D., Rudney, J.D., Sparks, B.S. and Hodges, J.S. (2000) Attachment level loss associated with persistent presence of Bacteroides forsythus. *J Dent Res* **79**: 217.

Tran, S.D., Rudney, J.D., Sparks, B.S. and Hodges, J.S. (2001) Persistent presence of *Bacteroides forsythus* as a risk factor for attachment loss in a population with low prevalence and severity of adult periodontitis. *J Periodontol* **72**: 1–10.

Umeda, M., Ishikawa, I., Benno, Y. and Mitsuoka, T. (1990) Improved detection of oral spirochetes with an anaerobic culture method. *Oral Microbiol Immunol* **5**: 90–94.

Veith, P.D., Dashper, S.G., O'Brien-Simpson, N.M. *et al.* (2009) Major proteins and antigens of *Treponema denticola*. *Biochim Biophys Acta* **1794**: 1421–1432.

Verma, R.K., Bhattacharyya, I., Sevilla, A. *et al.* (2010a) Virulence of major periodontal pathogens and lack of humoral immune protection in a rat model of periodontal disease. *Oral Dis* **16**: 686–695.

Verma, R.K., Rajapakse, S., Meka, A. *et al.* (2010b) *Porphyromonas gingivalis* and *Treponema denticola* mixed microbial infection in a rat model of periodontal disease. *Interdiscip Perspect Infect Dis* **2010**: 605125.

Washizu, M., Ishihara, K., Honma, K. and Okuda, K. (2003) Effects of a mixed infection with *Porphyromonas gingivalis* and *Treponema denticola* on abscess formation and immune responses in mice. *Bull Tokyo Dent Coll* **44**: 141–147.

Yoneda, M., Hirofuji, T., Anan, H. *et al.* (2001) Mixed infection of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a murine abscess model: involvement of gingipains in a synergistic effect. *J Periodontal Res* **36**: 237–243.

Zubery, Y., Dunstan, C.R., Story, B.M. *et al.* (1998) Bone resorption caused by three periodontal pathogens *in vivo* in mice is mediated in part by prostaglandin. *Infect Immun* **66**: 4158–4162.