Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 605125, 10 pages
doi:10.1155/2010/605125

Research Article

Porphyromonas gingivalis and *Treponema denticola* Mixed Microbial Infection in a Rat Model of Periodontal Disease

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Received 21 January 2010; Accepted 11 March 2010

Academic Editor: Bettina Fries

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Porphyromonas gingivalis and *Treponema denticola* are periodontal pathogens that express virulence factors associated with the pathogenesis of periodontitis. In this paper we tested the hypothesis that *P. gingivalis* and *T. denticola* are synergistic in terms of virulence; using a model of mixed microbial infection in rats. Groups of rats were orally infected with either *P. gingivalis* or *T. denticola* or mixed microbial infections for 7 and 12 weeks. *P. gingivalis* genomic DNA was detected more frequently by PCR than *T. denticola*. Both bacteria induced significantly high IgG, IgG2b, IgG1, IgG2a antibody levels indicating a stimulation of Th1 and Th2 immune response. Radiographic and morphometric measurements demonstrated that rats infected with the mixed infection exhibited significantly more alveolar bone loss than shaminfected control rats. Histology revealed apical migration of junctional epithelium, rete ridge elongation, and crestal alveolar bone resorption; resembling periodontal disease lesion. These results showed that *P. gingivalis* and *T. denticola* exhibit no synergistic virulence in a rat model of periodontal disease.

1. Introduction

Periodontitis is a chronic immunoinflammatory infectious disease leading to the destruction of periodontal ligament and adjacent supportive alveolar bone induced by pathogenic biofilms containing numerous periodontal pathogens. Among the periodontal pathogens, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are commonly co-isolated in subgingival biofilm samples from adult periodontitis lesions [1–5]. This consistent coexistence suggests that a strong ecological relationship may exist among these microbial species. Furthermore, several studies report the co-existence of *P. gingivalis* and *T. denticola* in close association with chronic periodontitis lesions [1, 6–8], detection in carotid and aortic atheromatous plaques [9], exhibit nutritional interactions [10], demonstrate bimodal co-aggregation [11–13], binding of *P. gingivalis* fimbriae to *T. denticola* dentilisin [14], as well as synergistic biofilm formation [15, 16]. Moreover, both species express high trypsin-like proteolytic enzyme activities [17–20] in addition to synergistic virulence as mixed infections in mouse abscess [21, 22] and pneumonia animal models [23]. We have shown previously that *P. gingivalis* and *Fusobacterium nucleatum* exhibit synergistic soft tissue destruction [24].

The mechanisms of interaction between *P. gingivalis* and *T. denticola* as a consortium in the subgingival sulcus and whether they express a synergistic pathogenic potential in progressing periodontitis remain enigmatic at present [19]. Recently, we have demonstrated that *P. gingivalis*, *T. denticola*, and *T. forsythia* with and without *F. nucleatum* not only exist as a consortium that is associated with chronic periodontitis in humans but also exhibit virulence resulting in the colonization of the rat oral cavity, induction of enhanced IgG immune responses, and significant alveolar bone resorption characteristic of polymicrobial (three pathogens or more) periodontitis [25].

Monomicrobial (single pathogen) periodontal infections using *P. gingivalis*, *T. denticola*, *T. forsythia*, or *F. nucleatum*

have been studied in rats and mice [25–30]. Increasing evidence supports the concept that bacterial interactions among members of the subgingival pathogens at any time during periodontal disease progression are important. However, there are no published reports establishing a mixed microbial (two species) periodontal infection for examining the virulence between *P. gingivalis* and *T. denticola*. This study examined mixed microbial periodontal disease using *P. gingivalis* and *T. denticola* as a consortium and examined their colonization/infection characteristics, periodontal inflammation parameters, immune response patterns, induction of alveolar bone resorption, and virulence interactions.

2. Materials and Methods

2.1. Bacterial Strains and Inocula. The bacteria used in this study were *P. gingivalis* 381 and *T. denticola* ATCC 35404 and these strains were grown under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) at 37°C in a Coy anaerobic chamber as described previously [25, 31]. The *P. gingivalis* strain 381 was chosen due to its known role in alveolar bone resorption in adult periodontitis and its proven ability to colonize the oral cavity of rodents [25, 27, 28, 32]. For oral monobacterial infection, *P. gingivalis* (2×10^{10} cells per mL: grown for 3 days on CDC anaerobic 5% sheep blood agar plates) or *T. denticola* (2×10^{10} cells per mL: grown in GM-1 broth for 48–72 hours as a log phase culture) was mixed with equal volumes of sterile 2% (w/v) low viscosity carboxymethylcellulose with PBS (CMC: Sigma), [25, 26, 28, 33] and one mL was used for infection (10^{10} cells per mL) by oral gavage [25, 26, 28, 33]. For oral mixed microbial infection, *P. gingivalis* (2×10^{10} cells per mL) was gently mixed with an equal volume of *T. denticola* (2×10^{10} cells per mL), mixed gently for 1-2 min, and allowed to interact for additional 5 minutes for any interactions among these species. An equal volume of sterile 2% (w/v) CMC was added, mixed thoroughly, and one mL (5×10^9 cells of *P. gingivalis* mL, 5×10^9 cells of *T. denticola*) was administered by oral gavage and anal topical application. Rats are coprophagic in nature, and the rationale behind the anal application is that bacteria will be in the feces and will then return to the oral cavity, thereby establishing a cycle of oral reinfection [28, 34].

2.2. Rat Oral Infections. Female Sprague-Dawley rats (8 weeks old, Charles River laboratories, MA, USA) were maintained in groups and housed in microisolator cages in an AALAC facility at the University of Florida. The protocol (#E900) and all rat infection procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Florida. Animals were fed standard powdered chow (Teklad Global 18% protein rodent diet 2918, Harlan) and water *ad libitum*. All rats were administered kanamycin (20 mg) and ampicillin (20 mg) daily for 4 days in the drinking water [25, 35] and the oral cavity was swabbed with 0.12% (v/v) chlorhexidine gluconate (Proctor and Gamble, OH, USA) mouth rinse [25, 31] to inhibit the endogenous organisms and to promote subsequent colonization of *P. gingivalis* and *T. denticola*. Rats were randomized into groups ($n = 18$) and monobacterial and mixed microbial inocula were administered by oral gavage and anal topical application [28, 34] for 4 consecutive days per week on 4–6 alternate weeks (16–24 inoculations). Sham-infected control rats received vehicle and sterile 2% low viscosity CMC only.

2.3. Oral Microbial Sampling. Oral microbial samples from rats were collected using sterile cotton swabs at pre- and post-infections. A total of 4–6 postinfection microbial samples were collected the following week from all the infected rats. To determine the kinetics of virulence of mono- and mixed infection-induced periodontal disease and immune responses, rats were euthanized at the beginning of the 8th (7 weeks of 16 inoculations) and 13th weeks (12 weeks of 24 inoculations). Blood was collected and sera were stored at −20°C for IgG antibody analysis. The rats were killed, skulls were removed, autoclaved, and mechanically defleshed with a periodontal scaler. The randomly selected rat jaws ($n = 3$) were also suspended in 10% (v/v) buffered formalin, decalcified, tissues trimmed, and used for histomorphometry and histology.

2.4. Monitoring Bacterial Colonization/Infection. DNA was isolated from rat oral microbial samples using a Wizard Genomic DNA purification kit (Promega, WI, USA). The standard genomic DNA for *P. gingivalis*, and *T. denticola* were also extracted following the same procedure from their respective 24–72 hours pure cultures as described previously in [25]. Subsequently, PCR was performed using 16S rRNA gene species-specific PCR oligonucleotide primers with a Bio-Rad thermal cycler as described previously in [25, 31, 35]. After amplification; PCR products were separated by 1.5% agarose gel electrophoresis and the bands were visualized using the UVP BioDoc-It Imaging System. The genomic DNA extracted from *P. gingivalis* and *T. denticola* served as positive control and PCR performed with no template DNA making up the negative control. Each PCR assay with the standard DNA was sensitive enough to detect 0.05 pg of DNA (*P. gingivalis* 30 cells; *T. denticola* 18 cells). Different PCR cycles from 35 to 40 were standardized to produce detectable amplicons with the least amount (0.05 pg) of template DNA.

2.5. Antibody Analysis. Blood was collected from each rat at the time of euthanasia. Serum from monobacterial ($n = 9$) or mixed microbial infected rats ($n = 9$) at 7 weeks (4 infections) and 12 weeks (6 infections) was used to determine IgG, IgM, IgA, and IgG subclass (IgG1, IgG2a, IgG2b, IgG2c) antibody concentrations, using a standard ELISA protocol [25, 34–36]. Briefly, diluted infected rat serum (1 : 100 for IgG and 1 : 20 for IgM, IgA and IgG subclass) was incubated in wells of either *P. gingivalis* or *T. denticola* coated microtiter polystyrene plates (Costar, Corning, NY, USA) for 2 hours at room temperature. After washing, alkaline phosphatase-conjugated goat anti-rat IgG (1 : 2000), IgM and IgA (Bethyl Laboratories, TX, USA) were added (1 : 500) and incubated

for additional 2 h at room temperature on a rotator. The substrate (*p*-nitrophenylphosphate; Sigma, 1 mg/ml) was added to the washed plates, and the reaction was terminated by using 3 M NaOH. For IgG subclass ELISA analysis, alkaline phosphatase-conjugated goat anti-rat IgG1, IgG2a, IgG2b, and IgG2c (Bethyl Laboratories, TX, USA) were used (1 : 500). The optical density (OD) was measured at OD_{405} nm using a Bio-Rad Microplate Reader. The infected rat serum antibody concentration was quantified using a gravimetric standard curve. The standard curve consisted of 8-rat IgG concentrations (Sigma, St. Louis, USA), which were coated onto wells of the microtiter polystyrene plates, detected, and developed as described above.

2.6. Morphometry Analysis of Horizontal Alveolar Bone Resorption. The pattern of alveolar bone resorption (horizontal or vertical) induced by *P. gingivalis* and *T. denticola* were measured by both morphometric and radiograph methods, respectively. The 7 and 12 weeks mono- and mixed microbial infected jaws ($n = 6$) were immersed in 3% (vol/vol) hydrogen peroxide overnight, and stained with 0.1% (wt/vol) methylene blue to delineate the cemento-enamel junction (CEJ) using the modified morphometric method [33, 34]. The digital images of both buccal and lingual root surfaces of all molar teeth were captured under a 10 × stereo dissecting microscope (SteReo Discovery V8; Carl Zeiss), after superimposition of buccal and lingual cusps to ensure reproducibility and consistency. The line tool was used to make horizontal bone resorption measurements on all molars in each quadrant from the CEJ to the alveolar bone crest (ABC). The surface perimeters of CEJ and ABC were traced using the calibrated line tool. As the AxioVision software program was calibrated using a precise ruler, the area of the horizontal bone loss reading in mm^2 is instantly imprinted over the digital image. Two blinded investigators were used and all measurements were done two times by the same examiner at separate times and the means of the measurements were obtained for each of the four quadrants.

2.7. Radiographic Assessment of Vertical Alveolar Bone Loss. The maxillae and mandibles were placed and stabilized with dental wax on a digital Kodak 6000 sensor (CareStream Health, USA) oriented with the axis of the teeth parallel to the sensor surface. Digital radiographs of distal and mesial surfaces of the molars were acquired with orthogonal projection geometry using an exposure time of 0.08 s at 60 kVp and 15 mA. All radiographic images were exported into the Tuned Aperture Computed Tomography workbench, calibrated for magnification using known anatomic measurements, and histograms equalized. The line tool was used to make vertical bone resorption measurements on the distal and mesial sides of each interproximal surface (2 sites per tooth) for each of the molars in each quadrant from the CEJ to the ABC (i.e., resorption) as the primary outcome parameter of the study. The summation of alveolar bone resorption in mm was tabulated and analyzed for intra and intergroup comparison [25, 31, 35].

2.8. Histomorphometric Analysis of Periodontal Tissue. Monomicrobial and mixed microbial infected rat jaws ($n = 3$) were removed randomly and fixed in 10% buffered formalin. Bone was decalcified in Immunocal (Decal Chemical) for 28 days at 4°C. The decalcified tissue was embedded in paraffin blocks, 4 μ sections prepared, stained with hematoxylin and eosin and the wholeslides were digitally scanned with a ScanScope CS system (Aperio Technologies, Vista, CA). The scanned slides were viewed with ImageScope viewing software (Aperio Technologies, Vista, CA). The inflammation at the supracrestal gingival connective tissue between the molars in each specimen at consecutive sections or levels 10 and 20 was examined based on multiple parameters including polymorphonuclear leukocytes (PMN), lymphocytes, blood vessel density, apical migration of junctional epithelium (JE), rete ridge elongation (R) and alveolar bone resorption (ABR) [37]. The number of inflammatory cells (PMNs and lymphocytes) per unit area (0.05 mm × 0.05 mm) was counted in the area of the junctional epithelium and adjacent connective tissue [37]. The migration of JE, elongation of rete ridges and resorption of alveolar bone was measured using the ImageScope software with a microgrid at a magnification of ×200. The distances from the CEJ to the coronal portion of the connective tissue attachment (apical migration), from the CEJ to the apical portion of the rete ridge; and from the CEJ to the level of the alveolar bone crest were measured [37].

2.9. Statistical Analyses. The alveolar bone resorption and IgG antibody data were presented as means ± standard deviations (Prism 4, GraphPad software). *P* values were calculated using the Kruskal Walis ANOVA with Dunn's correction for multiple comparisons and Mann-Whitney Student *T*-test [37]. *P* values of .05 were considered statistically significant.

3. Results

3.1. Induction of Periodontal Disease. Prior to mono- or mixed infection; we examined all rats for *P. gingivalis* and *T. denticola* using appropriate bacterium-specific primers by PCR, and observed all rats were consistently negative for these oral pathogens. The PCR results demonstrate an appropriately sized amplicon for *P. gingivalis* (600 bp) present in DNA isolated from rat oral microbial samples following infection with the single-microbe inocula (data not shown). Among two monobacterial infections, *P. gingivalis* was found positive by PCR in all individual rats ($n = 18$) and was positive 1–4 times during the four to six sampling times. In addition, 50–83.3% of infected rats were positive for *P. gingivalis* during four (2, 3, 4, and 6) out of six sampling times. In contrast, *T. denticola* (860 bp) amplicons were negative for DNA isolated from rat oral microbial samples following infection with the single-microbe inocula. The rats that had been given *P. gingivalis* + *T. denticola* mixed infection showed 72% (13/18) positive amplicons for *P. gingivalis* and only 2 out of 18 rats were positive for amplicons of *T. denticola*.

3.2. Serum IgG Antibody to Oral Infections. To provide additional documentation of oral infection and to demonstrate an immunological response to *P. gingivalis* and *T. denticola* infection, we evaluated the levels of pathogen specific IgG, IgM, IgA, and IgG subclass (IgG1, IgG2a, IgG2b, IgGc) antibodies in rat sera 7 weeks and 12 weeks post-initial infection (Figure 1). The induction of IgG antibody response patterns was identical in both 7 and 12 weeks infected rats using either mono- or mixed infection protocols. All rats in the *P. gingivalis* infected group at both 7 (Figure 1(a)) and 12 weeks (Figure 1(c)) demonstrated significantly elevated IgG antibody ($P < .05$) compared to the levels in shaminfected control rats. Similarly, all rats infected with *T. denticola* produced IgG antibody that was significantly higher ($P < .05$) than levels of sham-infected control rats at both 7 (Figure 1(b)) and 12 weeks (Figure 1(e)) postinfection. However, *P. gingivalis* infection induced significantly higher IgG levels (>100-fold) than *T. denticola* infection in the monoinfected rats. The levels of IgG antibody in the rat serum paralleled the frequency of detection of *P. gingivalis* in the oral microbial samples. Interestingly, all rats in the *T. denticola* monobacterial infection group at 12 weeks (Figure 1(e)) induced significantly strong IgG immune response in spite of our inability to detect *T. denticola* DNA in the oral microbial samples.

All rats in the mixed infection groups showed elevated serum IgG antibodies to *P. gingivalis* and *T. denticola* compared to the levels in shaminfected control rats (Figure 1). Interestingly, *P. gingivalis* + *T. denticola* infection at 7 (Figures 1(a) and 1(b)) and 12 week (Figures 1(d) and 1(f)) post-initial infection induced >100-fold stronger *P. gingivalis* specific IgG immune response as compared to *T. denticola* IgG responses. Approximately, 90% of the rats infected with mixed bacteria (16/18) presented elevated serum IgG to *P. gingivalis* when compared to sham-infected control rats (Figure 1(a)). Similarly, 100% of the mixed microbial infected rats (18/18) demonstrated significantly elevated serum IgG to *T. denticola* compared to that in sham-infected control rats (Figure 1(b)). None of the *P. gingivalis* and *T. denticola* infected rats induced IgA and IgM antibodies during 7 and 12 weeks of infection (data not shown).

3.3. IgG Subclass Responses to Infection. Thus, we had observed that *P. gingivalis* and *T. denticola* are antigenic in the rats, which resulted in significant levels of serum IgG antibodies. In order to more fully assess the characteristics of the antibody, we determined the IgG subclass distribution of the humoral immune response following oral infection. Following 12 weeks of *P. gingivalis* mono- and mixed microbial infection, the IgG2b subclass levels ($P < .05$) were higher than the IgG1 and IgG2a antibody levels and significantly ($P < .05$) greater than in sham-infected control rats (Figures 1(c) and 1(d)). Similarly, in *T. denticola* mono- and mixed microbial infection, the IgG2b subclass levels ($P < .05$) were higher than the IgG1 and IgG2c antibody levels and significantly greater than in sham-infected control rats ($P < .05$) (Figures 1(e) and 1(f)). Additionally, *P. gingivalis* induced higher levels of IgG subclass antibody than that induced by *T. denticola*.

3.4. Morphometric Evaluation of Alveolar Bone Loss. In order to address the potential virulence between the *P. gingivalis* and *T. denticola* in periodontal disease progression in the rats, we examined the effect of infection on the maxilla and mandible alveolar bone resorption. Both mono- and mixed infection induced buccal and palatal areas of alveolar bone resorption (Table 1; Figure 2). Here, the maxillary and mandibular bone loss in the rats infected with *P. gingivalis*, *T. denticola*, and *P. gingivalis* + *T. denticola* were significantly greater ($P < .05$) than that of the sham-infected control group at 7 and 12 weeks (Table 1). The maxillary and mandibular bone loss in all infected groups during 12 weeks of periodontal disease was generally greater than 7 weeks of periodontal disease. In addition, mandibular bone loss was generally higher than maxillary bone loss in both buccal and palatal surfaces (Table 1). *P. gingivalis* monoinfection induced more palatal mandibular horizontal bone loss area than maxilla at 7 and 12 weeks post-initial infection. In contrast, *T. denticola* monoinfection generally induced greater palatal and buccal area bone loss than *P. gingivalis* as well as more mandibular bone loss than maxilla at 7 and 12 weeks post-initial infection. Similarly, mixed infection induced more significant bone loss in both maxilla and mandibles, palatal and buccal surfaces than monoinfection at 7 and 12 weeks post initial infection (Table 1). Furthermore, mixed infection induced more mandibular palatal surface horizontal area bone loss than maxillae.

3.5. Radiographic Evaluation of Interproximal Alveolar Bone Loss. In order to confirm our observations of alveolar bone resorption, radiographic analysis of the maxilla and mandible was performed. Mono- and mixed infection resulted in significantly increased maxillary, mandibular, and total interproximal alveolar bone resorption at 7 weeks (data not shown) and 12 weeks of periodontal disease compared with sham-infected control rats ($P < .05$) (Figure 3). In addition, there was an overall loss in vertical bone height, with associated circumferential angular defects. Furthermore, mixed infection demonstrated a significant increase in maxillary, mandibular, and total vertical bone loss compared to any of the monobacterial and sham-infected control infections ($P < .05$) (Figure 3).

3.6. Histological and Histometrical Analysis. In order to determine if the infection protocols induced differing levels of inflammation which could be responsible for the increased alveolar bone resorption observed, sections of the maxilla and mandible of rats infected with *P. gingivalis* and/or *T. denticola* during 7 and 12 weeks of periodontal disease were examined at consecutive levels 1, 10, and 20 for inflammation. Mixed microbial infection rats showed more significant histological changes, particularly apical migration of JE, rete ridge elongation, PMN density, lymphocytes infiltration, blood vessel density and alveolar bone loss

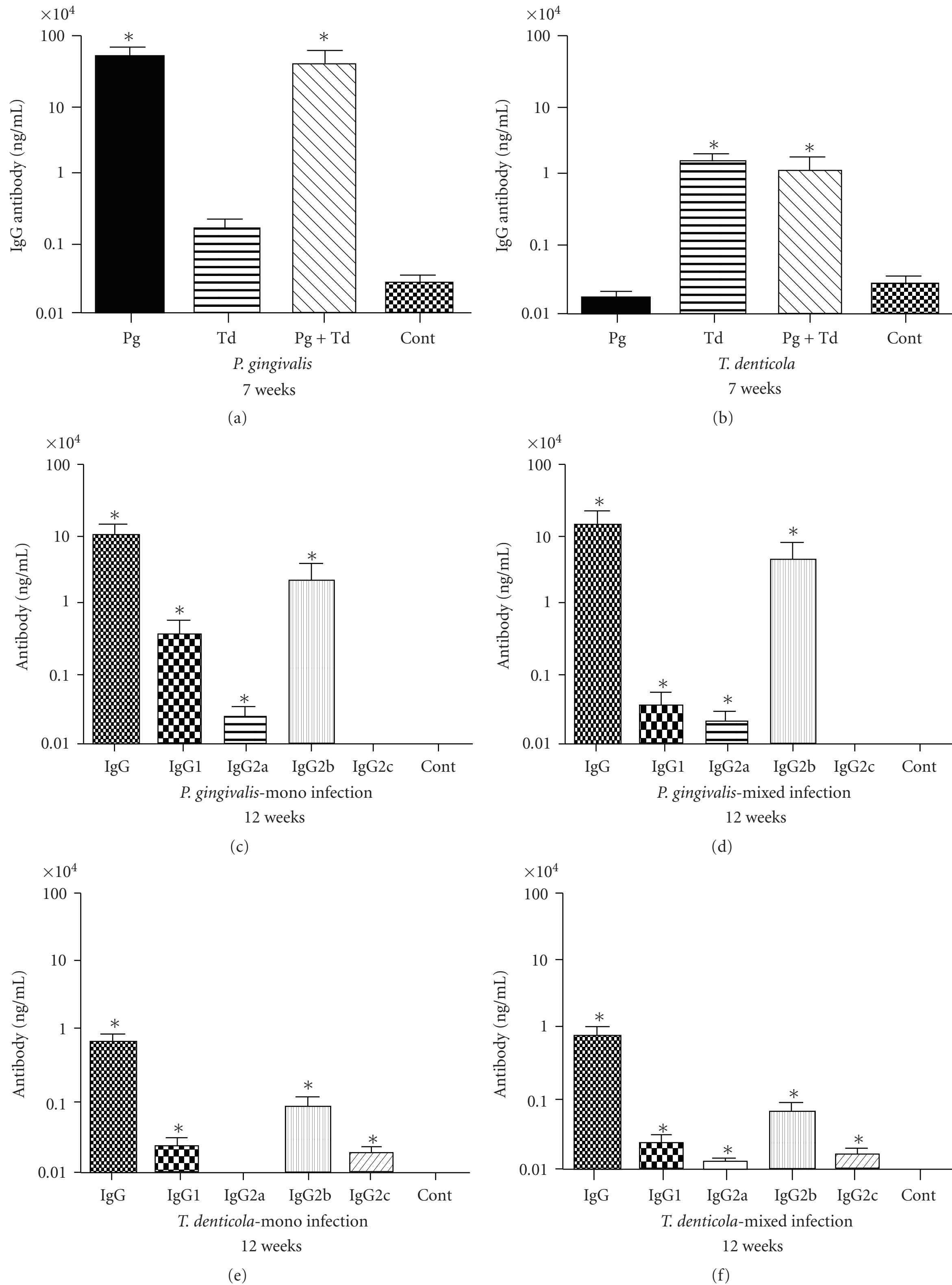


Figure 1: Serum IgG and IgG subclass (IgG1, IgG2a, IgG2b, IgGc) antibody levels. Serum IgG antibody levels in serum from rats [collected at end of a 7 weeks (a and b) and 12 weeks (c–f) infection] following mono infection ($n = 9$) or mixed infection ($n = 9$). The graphs show the results for IgG and IgG subclass antibody reactive with each of the two species of bacteria. The bars indicate the mean antibody concentrations in serum from rats orally infected with the individual bacteria or with mixed bacteria or from sham-infected control rats. The error bars indicate one standard deviation from the mean. An asterisk indicates that a value is significantly different ($^*P < .05$) than the value for sham-infected control rats or for antibody in serum from rats infected with a different microorganism. *Pg*, *P. gingivalis*; *Td*, *T. denticola*; Cont; sham-infected control.

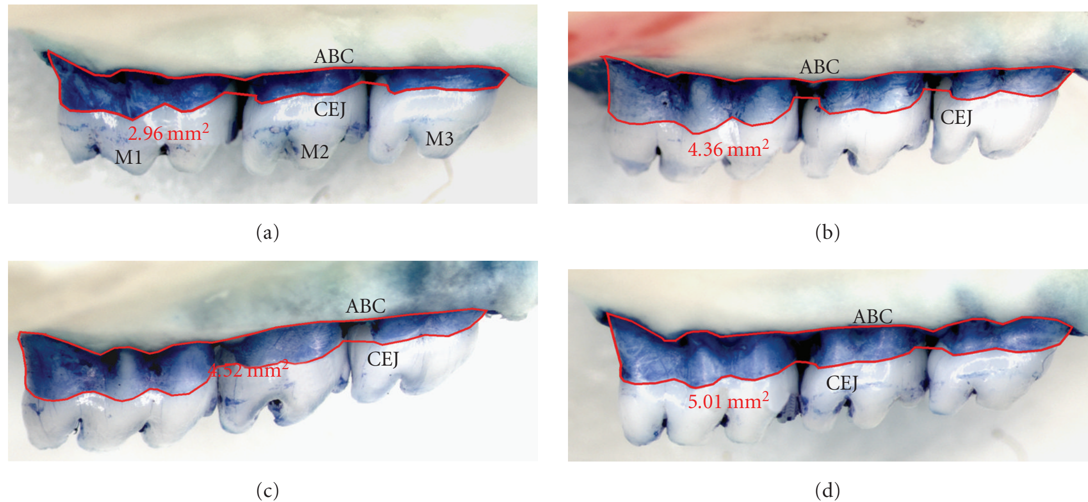


Figure 2: Morphometric evaulation of alveolar bone loss. Representative rat left maxilla of a sham-infected control rat (a) showing the buccal horizontal bone loss area by morphometry. The area outlined between CEJ-ABC represents the area of horizontal alveolar bone resorption in mm^2 (b). Rat left maxilla infected with *P. gingivalis*, (c) *T. denticola*, and (d) *P. gingivalis* + *T. denticola* during 12 weeks of periodontal disease showing extensive horizontal alveolar bone loss. M1, M2, and M3 are molars. Rat jaw images captured at 10× magnification.

Table 1: Morphometric measurements of horizontal alveolar bone loss (area) on the buccal and palatal surfaces of all molars in rats.

Infection/Groups		Control	*P. gingivalis*	*T. denticola*	*P. gingivalis* + *T. denticola*
Maxilla 7 Weeks	Buccal	3.2 ± 0.45*	3.4 ± 0.47[a]	4.8 ± 0.47[b,c]	4.3 ± 0.45[b]
	Palatal	5.5 ± 0.47	6.2 ± 0.66[a]	6.5 ± 0.23[b]	7.5 ± 0.77[b,c,e]
Mandible 7 Weeks	Buccal	3.7 ± 0.38	4.5 ± 0.51[b]	4.4 ± 0.46[a]	4.8 ± 0.32[b]
	Palatal	6.5 ± 0.54	7.4 ± 0.51[a]	8.0 ± 0.4[b]	8.8 ± 0.8[b,d,e]
Maxilla 12 Weeks	Buccal	3.2 ± 0.45	5.1 ± 0.88[b]	5.8 ± 0.91[b]	5.4 ± 0.48[b]
	Palatal	5.6 ± 0.63	7.4 ± 0.98[b]	7.7 ± 0.74[b]	8.5 ± 0.64[b,c,e]
Mandible 12 Weeks	Buccal	3.5 ± 0.25	3.6 ± 0.64	4.7 ± 0.58[b,d]	4.2 ± 0.69[a]
	Palatal	6.6 ± 0.34	7.6 ± 0.80[b]	8.7 ± 0.51[b,d]	9.3 ± 0.33[b,d,e]

*Mean value and standard deviation from six mice per each group measured using AxioVision line tool software as described in methods section. [a]Significantly more than sham-infected control group ($P < .05$). [b]Significantly more than sham-infected control group ($P < .01$). [c]Significantly more than *P. gingivalis* infected group ($P < .05$). [d]Significantly more than *P. gingivalis* infected group ($P < .01$). [e]Significantly more than *T. denticola* infected group ($P < .05$).

than sham-infected control animals (Table 2; Figure 4). The sham-infected control rats showed mild inflammation with fewer PMN and lymphocytes, no apical migration, and small rete ridge elongation. Similarly, mixed microbial infection induced significant apical migration of JE ($P < .01$) and dense inflammation in the periodontium compared to *P. gingivalis* monoinfected rats. In addition, mixed microbial infection rats showed no significant differences in rete ridge elongation, PMN density, lymphocytes infiltration, and blood vessel density compared to *P. gingivalis* and *T. denticola* mono-infected rats. *P. gingivalis* and *T. denticola* infected rats showed significant histological changes, specifically apical migration of JE, alveolar bone loss, PMN density, lymphocytes infiltration, and blood vessel density more than sham-infected control animals (Table 2; Figure 4). Moreover, *P. gingivalis* infected rats showed significant infiltration of lymphocytes ($P < .05$) and blood vessel density in the periodontium ($P < .001$) compared to mixed microbial infected rats (Table 2).

4. Discussion

This paper explicitly demonstrates an experimental model for mixed microbial infections in periodontal disease, documenting colonization/infection with the *P. gingivalis/T. denticola* consortium of oral microorganisms, with (kinetics) induction of periodontal inflammation at 7 and 12 weeks, generation of specific systemic IgG immune responses to the infecting pathogens, and stimulation of enhanced alveolar bone resorption in rats. These results also documented, for the first time, the virulence of mixed infections with *P. gingivalis* + *T. denticola* in a periodontal disease model. Bacterial synergism in progression from periodontal health to disease has been proposed but few studies have documented bacterial synergism due to the inherent complexity of the subgingival microflora [8, 38]. In the previous study [25], we had not examined the early induction of periodontal inflammation and assessment of both palatal and buccal horizontal bone loss. Here we demonstrate this early kinetics

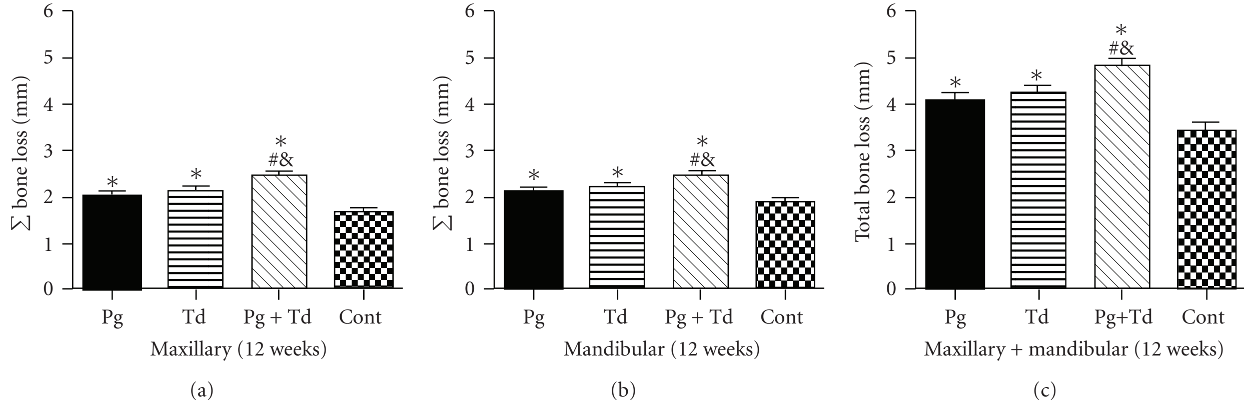


Figure 3: Interproximal evaluation of alveolar bone loss. Radiographic vertical alveolar bone resorption (12 weeks of periodontal disease) in rats ($n = 6$) following mono infection with *P. gingivalis* or *T. denticola* and with the mixed infection. Each bar indicates the mean interproximal alveolar bone loss for distance measured between CEJ and ABC at mesial and distal sites of three molar teeth (6 sites). The error bars indicate standard deviation from the mean. An asterisk sign denotes significantly different from sham-infected control rats ($P < .05$). The # sign indicates significant difference comparing between mono bacterial *P. gingivalis* and the mixed microbial infected rats ($P < .05$) and & sign indicates significant difference comparing between *T. denticola* and the mixed microbial infected rats ($P < .05$). *Pg*, *P. gingivalis*; *Td*, *T. denticola*; Cont; sham-infected control.

Table 2: Histometrical analysis of rat periodontal tissue after primary infection with *P. gingivalis* and *T. denticola* as mono- and mixed infection.

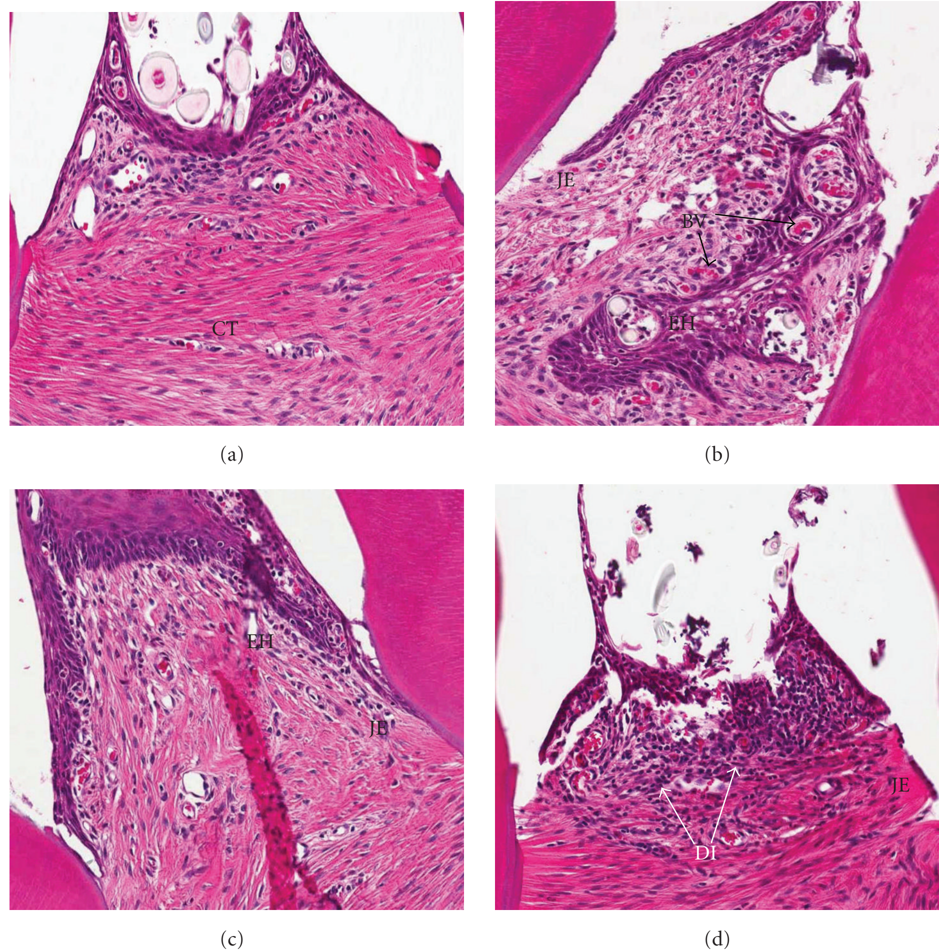
Histological Parameters	Control	*P. gingivalis*	*T. denticola*	*P. gingivalis* + *T. denticola*
Apical migration (μm)[†]	0 ± 0*	75.2 ± 79.5[b]	97.3 ± 59.9[b]	134.6 ± 66.5[b,d]
Rete ridge elongation (μm)[‡]	20.8 ± 18.4	68.6 ± 20.2	87 ± 39.1	101.8 ± 41.6[a]
Alveolar bone resorption (μm)[§]	205 + 15.9	357.9 + 108.8[a]	425.8 + 134.2[b]	451.2 + 68.4[b]
PMN density (number/0.05 mm × 0.05 mm)	2.1 ± 1.4	6.8 ± 2.9[c]	5 ± 2.1[b]	5.1 ± 2[b]
Lymphocytes	3.7 ± 0.9	11.7 ± 3.9[c,e]	5.5 ± 1.7[a]	6.6 ± 1.6[b]
Blood vessel density (number/0.05 mm × 0.05 mm)	2.1 ± 1.0	8.4 ± 1.9[c,f]	5 ± 2.5[b]	4.3 ± 1.7[b]

*Values are presented as mean ± standard deviation ($n = 5–9$). [†]The distances from the CEJ to the coronal portion of the connective tissue attachment (apical migration of JE). [‡]The distances from the CEJ to the apical portion of the rete ridge. [§]The distances from the CEJ to the level of the ABC. [a]Significantly more than sham-infected control group ($P < .05$). [b]Significantly more than sham-infected control group ($P < .01$). [c]Significantly more than sham-infected control group ($P < .0001$). [d]Significantly more than *P. gingivalis* group ($P < .01$). [e]Significantly more than *P. gingivalis* + *T. denticola* group ($P < .05$). [f]Significantly more than *P. gingivalis* + *T. denticola* group ($P < .0001$).

in a model of periodontal disease at 7 and 12 weeks of disease.

The monobacterial infection in rats indicated that *P. gingivalis* exhibited the ability to colonize/infect the oral cavity with 4–6 alternate weekly infection schedules (16–24 inoculations) during the 7–12 weeks study establishing a chronic infection. We have shown previously that infecting rats 15-16 times with *P. gingivalis*, over a similar interval of the experiment, resulted in consistent detection of genomic DNA in oral microbial samples [25, 31, 35]. Moreover, induction of significant IgG immune responses and enhanced alveolar bone loss observed in all rats clearly documents that these rats were infected, even though the rats showed negative PCR reactions for *T. denticola*. We recognize the limitations in sample collection procedures, as existing with all the current techniques of microbial sampling from the oral cavity.

The serum IgG antibody levels to monoinfection during 7 and 12 weeks of periodontal disease clearly indicated that *P. gingivalis* is highly effective in colonization and/or is highly antigenic in the rats when compared to *T. denticola*. The antibody responses demonstrated substantial specificity for each of the infecting species. However, we observed an increase in serum IgG antibody to *T. denticola* in *P. gingivalis* mono-infected rats. While this "nonspecific" IgG antibody was more than sham-infected controls, it was approximately 1000-fold lower than the homologous IgG antibody response to *P. gingivalis* infection. This could indicate that these bacteria share some common epitopes [25]. The mixed oral infection with *P. gingivalis/T. denticola* elicited somewhat different profiles of serum IgG antibodies. These altered responses could be due to a lowered colonization capacity of the *T. denticola* within the mixed consortium challenge and/or a decreased ability to multiply in the oral cavity during the infection, thus resulting in a lower magnitude of antigenic challenge, reduced periodontal inflammation, and no robust alveolar bone loss nor virulence synergism. The predominant response following *P. gingivalis* infection was the IgG2b (T helper type 1) and IgG1 subclass (T helper type 2), followed by IgG2a (Th1) and undetectable level



(a) (b) (c) (d)

Figure 4: Representative sections from histopathology of periodontal tissue. Comparative maxillary histology (hematoxylin and eosin) of alveolar bone sections from maxilla of rat infected with *P. gingivalis* and/or *T. denticola* at 12 weeks. (a) from sham-infected control rat displaying minimal inflammation, lack of migration of junctional epithelium (JE) and minimal inflammation in the connective tissue (CT); (b) from the *P. gingivalis* infected rat showing prominent epithelium hyperplasia (EH), migration of JE and increase in number of blood vessels (BV) (indicated by black arrows); (c) from the *T. denticola* infected rat also exhibits migration of JE and EH; and (d) from the mixed infection with *P. gingivalis* + *T. denticola* infected rat demonstrates dense inflammation (DI) (indicated by white arrows) along with migration of JE. JE indicates junctional epithelium, CT connective tissue, EH epithelial hyperplasia, BV blood vessels, DI dense inflammation. All images at 20× magnification.

of IgG2c antibody indicating a stimulation of both Th1 and Th2 activities in development of the humoral immune response to bacterial infection. Similarly, the predominant response following *T. denticola* monoinfection was the IgG2b subclass, followed by IgG1, IgG2c and undetectable level of IgG2a antibody in rats suggesting a mixed Th1- and Th2-responses to oral infection. In contrast, IgG1 antibody titer was much higher in mice to *T. denticola* infection [29]. Despite the high bacterial specific IgG antibody levels during 7 and 12 weeks of infection, there was no significant immune protection from alveolar bone loss in rats as well as in several previous studies [39, 40] suggesting complex mechanisms of antibody protection. Furthermore, *P. gingivalis* recombinant hemagglutinin B immunization or immunized and infected rats induced IgG subclass responses (IgG1 = IgG2a > IgG2b > IgG2c) suggesting a mixed Th1 and Th2 responses and immunized rats had less alveolar bone loss indicating a protective immune response [41, 42]. Similarly, immunization with *P. gingivalis* whole cells induced high-titer serum IgG2a (Th2), moderate-titer IgG2b (Th1) and low-titer IgG1 (Th2) responses and immunization with RgpA-Kgp cysteine proteases of *P. gingivalis* induced high-titer serum IgG2a (Th2) responses which restricted colonization and decreased periodontal bone loss indicating a protective immune response in the rat [34].

While differences in horizontal (palatal and buccal surface) and interproximal alveolar bone resorption levels were observed following monoinfection with *P. gingivalis* and *T. denticola* dependent upon both the differences in the sites of the samples as well as the techniques for measurements, we could not easily compare the magnitude of alveolar bone resorption between these individual bacteria. Importantly, in testing our hypothesis, oral infection with *P. gingivalis*/*T. denticola* significantly increased interproximal as well as

horizontal alveolar bone loss compared to mono-infections. This increased bone loss may be related to enhancement of expression of the virulence of individual bacteria by cooperative abilities of their extracellular potent proteinases (*P. gingivalis* RgpA, RgpB, Kgp gingipains cysteine proteinase) (*T. denticola* chymotrypsin-like protease, phospholipase C, oligopeptidase, endopeptidase and cystalysin) to affect host systems through specific cleavage of cell surface receptors and the inactivation of host-defense proteins [18, 20]. In addition, mixed infection with *P. gingivalis* + *T. denticola* exhibits significant virulence synergism in abscess formation and mortality in mouse pneumonia model [23] and mouse abscess model [21, 22].

5. Conclusions

The analysis of the data have clearly shown the following: (i) mono- and mixed microbial colonization/infection of human oral pathogens in rat oral cavity during 7 weeks of periodontal disease (gained access to the oral epithelium), (ii) generation of a specific serum IgG antibody responses (as early as 7 weeks) reflecting the oral infection (engagement of systemic host response mechanisms), (iii) induction of enhanced horizontal and interproximal alveolar bone resorption in rats with mixed infection as expected (direct result of local infection), (iv) induction of inflammatory response (apical migration of JE, rete ridge elongation, crestal alveolar bone loss, PMNs) consistent with established characteristics of periodontal disease, and (v) no synergistic virulence observed with *P. gingivalis/T. denticola* in a rat periodontal disease model. This mixed infection model will provide an opportunity for further studies to clarify the characteristics and alterations of the host response profiles such as proinflammatory cytokines and matrix metalloproteinases in periodontal tissues that relate to osteoclastic alveolar bone loss in response to mixed infections.

Acknowledgments

The authors thank Professor Howard Kuramitsu for critically reviewing this paper. This work was supported by USPHS research Grant U24 DE016509 (R. Burne) and DE-015720 (L. Kesavalu) from the National Institute of Dental and Craniofacial Research (NIDCR), NIH.

References

[1] S. S. Socransky, A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent Jr., "Microbial complexes in subgingival plaque," *Journal of Clinical Periodontology*, vol. 25, no. 2, pp. 134–144, 1998.

[2] S. S. Socransky and A. D. Haffajee, "Periodontal microbial ecology," *Periodontology 2000*, vol. 38, pp. 135–187, 2005.

[3] R. P. Ellen and V. B. Galimanas, "Spirochetes at the forefront of periodontal infections," *Periodontology 2000*, vol. 38, pp. 13–32, 2005.

[4] M. N. Sela, "Role of *Treponema denticola* in periodontal diseases," *Critical Reviews in Oral Biology and Medicine*, vol. 12, no. 5, pp. 399–413, 2001.

[5] S. C. Holt and J. L. Ebersole, "*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis," *Periodontology 2000*, vol. 38, pp. 72–122, 2005.

[6] T. Kigure, A. Saito, K. Seida, S. Yamada, K. Ishihara, and K. Okuda, "Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods," *Journal of Periodontal Research*, vol. 30, no. 5, pp. 332–341, 1995.

[7] G. R. Riviere, K. S. Smith, N. Carranza Jr., et al., "Associations between *Porphyromonas gingivalis* and oral treponemes in subgingival plaque," *Oral Microbiology and Immunology*, vol. 11, no. 3, pp. 150–155, 1996.

[8] L. G. Simonson, K. T. McMahon, D. W. Childers, and H. E. Morton, "Bacterial synergy of *Treponema denticola* and *Porphyromonas gingivalis* in a multinational population," *Oral Microbiology and Immunology*, vol. 7, no. 2, pp. 111–112, 1992.

[9] F. Cavrini, V. Sambri, A. Moter, et al., "Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aortic atheromatous plaques by FISH: report of two cases," *Journal of Medical Microbiology*, vol. 54, no. 1, pp. 93–96, 2005.

[10] D. Grenier, "Nutritional interactions between two suspected periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*," *Infection and Immunity*, vol. 60, no. 12, pp. 5298–5301, 1992.

[11] D. Grenier, "Demonstration of a bimodal coaggregation reaction between *Porphyromonas gingivalis* and *Treponema denticola*," *Oral Microbiology and Immunology*, vol. 7, no. 5, pp. 280–284, 1992.

[12] P. E. Kolenbrander, "Oral microbial communities: biofilms, interactions, and genetic systems," *Annual Review of Microbiology*, vol. 54, pp. 413–437, 2000.

[13] M. Onagawa, K. Ishihara, and K. Okuda, "Coaggregation between *Porphyromonas gingivalis* and *Treponema denticola*," *The Bulletin of Tokyo Dental College*, vol. 35, no. 4, pp. 171–181, 1994.

[14] M. Hashimoto, S. Ogawa, Y. Asai, Y. Takai, and T. Ogawa, "Binding of *Porphyromonas gingivalis* fimbriae to *Treponema denticola* dentilisin," *FEMS Microbiology Letters*, vol. 226, no. 2, pp. 267–271, 2003.

[15] H. K. Kuramitsu, W. Chen, and A. Ikegami, "Biofilm formation by the periodontopathic bacteria *Treponema denticola* and *Porphyromonas gingivalis*," *Journal of Periodontology*, vol. 76, pp. 2047–2051, 2005.

[16] M. Yamada, A. Ikegami, and H. K. Kuramitsu, "Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis*," *FEMS Microbiology Letters*, vol. 250, no. 2, pp. 271–277, 2005.

[17] S. C. Holt, L. Kesavalu, S. Walker, and C. A. Genco, "Virulence factors of *Porphyromonas gingivalis*," *Periodontology 2000*, vol. 20, no. 1, pp. 168–238, 1999.

[18] J. C. Fenno and B. C. McBride, "Virulence factors of oral treponemes," *Anaerobe*, vol. 4, no. 1, pp. 1–17, 1998.

[19] R. J. Lamont and H. F. Jenkinson, "Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 4, pp. 1244–1263, 1998.

[20] J. Potempa, A. Banbula, and J. Travis, "Role of bacterial proteinases in matrix destruction and modulation of host responses," *Periodontology 2000*, vol. 24, no. 1, pp. 153–192, 2000.

[21] L. Kesavalu, S. C. Holt, and J. L. Ebersole, "Virulence of a polymicrobic complex, *Treponema denticola* and *Porphyromonas gingivalis*, in a murine model," *Oral Microbiology and Immunology*, vol. 13, no. 6, pp. 373–377, 1998.

[22] M. Washizu, K. Ishihara, K. Honma, and K. Okuda, "Effects of a mixed infection with *Porphyromonas gingivalis* and *Treponema denticola* on abscess formation and immune responses in mice," *The Bulletin of Tokyo Dental College*, vol. 44, no. 3, pp. 141–147, 2003.

[23] R. Kimizuka, T. Kato, K. Ishihara, and K. Okuda, "Mixed infections with *Porphyromonas gingivalis* and *Treponema denticola* cause excessive inflammatory responses in a mouse pneumonia model compared with monoinfections," *Microbes and Infection*, vol. 5, no. 15, pp. 1357–1362, 2003.

[24] J. L. Ebersole, F. Feuille, L. Kesavalu, and S. C. Holt, "Host modulation of tissue destruction caused by periodontopathogens: effects on a mixed microbial infection composed of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*," *Microbial Pathogenesis*, vol. 23, no. 1, pp. 23–32, 1997.

[25] L. Kesavalu, S. Sathishkumar, V. Bakthavatchalu, et al., "Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease," *Infection and Immunity*, vol. 75, no. 4, pp. 1704–1712, 2007.

[26] P. J. Baker, L. DuFour, M. Dixon, and D. C. Roopenian, "Adhesion molecule deficiencies increase *Porphyromonas gingivalis*-induced alveolar bone loss in mice," *Infection and Immunity*, vol. 68, no. 6, pp. 3103–3107, 2000.

[27] B. Klausen, "Microbiological and immunological aspects of experimental periodontal disease in rats: a review article," *Journal of Periodontology*, vol. 62, no. 1, pp. 59–73, 1991.

[28] E. Lalla, I. B. Lamster, M. A. Hofmann, et al., "Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 8, pp. 1405–1411, 2003.

[29] S. F. Lee, E. Andrian, E. Rowland, and I. C. Marquez, "Immune response and alveolar bone resorption in a mouse model of *Treponema denticola* infection," *Infection and Immunity*, vol. 77, no. 2, pp. 694–698, 2009.

[30] S. F. Lee, E. Andrian, E. Rowland, and I. C. Marquez, "Immune response and alveolar bone resorption in a mouse model of *Treponema denticola* infection," *Infection and Immunity*, vol. 77, no. 2, pp. 694–698, 2009.

[31] S. Sathishkumar, A. Meka, D. Dawson, et al., "Extracorporeal shock wave therapy induces alveolar bone regeneration," *Journal of Dental Research*, vol. 87, no. 7, pp. 687–691, 2008.

[32] R. T. Evans, B. Klausen, N. S. Ramamurthy, L. M. Golub, C. Sfintescu, and R. J. Genco, "Periodontopathic potential of two strains of *Porphyromonas gingivalis* in gnotobiotic rats," *Archives of Oral Biology*, vol. 37, no. 10, pp. 813–819, 1992.

[33] J.-Y. Lee, N.-N. Yi, U.-S. Kim, J.-S. Choi, S.-J. Kim, and J.-I. Choi, "*Porphyromonas gingivalis* heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontopathogenic bacteria," *Journal of Periodontal Research*, vol. 41, no. 1, pp. 10–14, 2006.

[34] P. S. Rajapakse, N. M. O'Brien-Simpson, N. Slakeski, B. Hoffmann, and E. C. Reynolds, "Immunization with the RgpA-Kgp proteinase-adhesin complexes of *Porphyromonas gingivalis* protects against periodontal bone loss in the rat periodontitis model," *Infection and Immunity*, vol. 70, no. 5, pp. 2480–2486, 2002.

[35] L. Kesavalu, B. Vasudevan, B. Raghu, et al., "Omega-3 fatty acid effect on alveolar bone loss in rats," *Journal of Dental Research*, vol. 85, no. 7, pp. 648–652, 2006.

[36] L. Kesavalu, S. C. Holt, and J. L. Ebersole, "Lack of humoral immune protection against *Treponema denticola* virulence in a murine model," *Infection and Immunity*, vol. 67, no. 11, pp. 5736–5746, 1999.

[37] D. Ekuni, T. Yamamoto, R. Yamanaka, K. Tachibana, and T. Watanabe, "Proteases augment the effects of lipopolysaccharide in rat gingiva," *Journal of Periodontal Research*, vol. 38, no. 6, pp. 591–596, 2003.

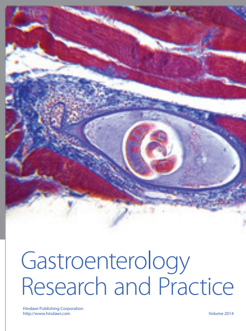
[38] K. A. Brogden, J. M. Guthmiller, and C. E. Taylor, "Human polymicrobial infections," *The Lancet*, vol. 365, no. 9455, pp. 253–255, 2005.

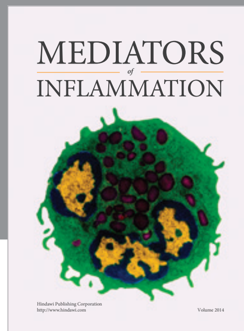
[39] P. J. Baker, S. Carter, M. Dixon, R. T. Evans, and D. C. Roopenian, "Serum antibody response to oral infection precedes but does not prevent *Porphyromonas gingivalis*-induced alveolar bone loss in mice," *Oral Microbiology and Immunology*, vol. 14, no. 3, pp. 194–196, 1999.

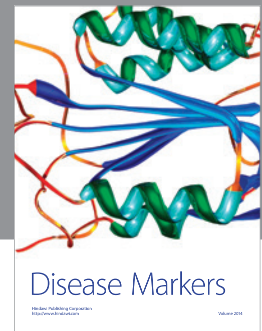
[40] A. A. DeCarlo, Y. Huang, C. A. Collyer, D. B. Langley, and J. Katz, "Feasibility of an HA2 domain-based periodontitis vaccine," *Infection and Immunity*, vol. 71, no. 1, pp. 562–566, 2003.

[41] J. Katz, K. P. Black, and S. M. Michalek, "Host responses to recombinant hemagglutinin B of *Porphyromonas gingivalis* in an experimental rat model," *Infection and Immunity*, vol. 67, no. 9, pp. 4352–4359, 1999.

[42] J. Katz and S. M. Michalek, "Effect of immune T cells derived from mucosal or systemic tissue on host responses to *Porphyromonas gingivalis*," *Oral Microbiology and Immunology*, vol. 13, no. 2, pp. 73–80, 1998.







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