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# Association of High Plaque Levels of *Prevotella intermedia* with Aggressive Periodontitis in a 16-year-old Female

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**Background:** Aggressive periodontitis (AP) encompasses a group of highly destructive forms of periodontitis. Its pathogenesis may be related to alterations in inflammatory and immune responses and in the oral flora.
**Methods:** A 16-year-old Black female patient presented with chief complaints of gingival pain and tooth mobility. She received a complete clinical dental examination, full mouth periapical radiographs, and laboratory tests including complete blood count, comprehensive metabolic panel, and microbiological plaque analysis. In vitro studies included salivary analysis and neutrophil and monocyte response to lipopolysaccharide (LPS).
**Results:** The patient had severe generalised gingival inflammation, type III tooth mobility, 8 to 10+ mm pocket depths, and extreme, generalised alveolar bone loss. Blood analysis revealed chronic non-cyclic neutropaenia, monocytosis, and slightly elevated protein levels. Subgingival plaque contained high levels of *Prevotella intermedia/nigrescens* (*P.i.*) (~50%) and *Porphyromonas gingivalis* (*P.g.*) (~15%). The patient's neutrophils and monocytes produced superoxide and interleukin-1β normally in response to *N*-formylmethionyl-leucyl-phenylalanine and *E. coli* LPS, and the neutrophils attached and migrated normally. *Prevotella* LPS had lower neutrophil priming activity than *Actinobacillus actinomycetemcomitans* or *Fusobacterium nucleatum* LPS. Salivary protein, and myeloperoxidase and lactoperoxidase activities were elevated.
**Conclusions:** This report supports others associating *P.i.* and *P.g.* with AP. The high levels of *P.i.* in the plaque are of particular interest, because *P.i.* LPS had low ability to prime neutrophils. This excessive growth of *P.i.* and *P.g.*, coupled with low numbers of normally functioning circulating neutrophils, may have contributed to the development of AP.

Key words: aggressive periodontitis, *Prevotella intermedia*, neutrophils, lipopolysaccharide (LPS)

## INTRODUCTION

Aggressive periodontitis (AP) comprises a group of highly destructive forms of periodontitis, formerly referred to as early-onset periodontitis (EOP) (prepubertal, juvenile, and rapidly progressive periodontitis) (Armitage, 1999). This AP classification has replaced terminologies that were age-dependent or required knowledge of the rate of progression of the disease process. While AP is a diverse group of diseases, altered immunoregulation may be common to all of them (Barrington et al, 2000). As in chronic periodontitis, certain pro-inflammatory cytokines may be involved in the extensive bone loss characteristic of AP. One of the primary features of some forms of AP is alteration in neutrophil functions, including chemotaxis, phagocytosis, superoxide production and bactericidal mechanisms (Cianciola et al, 1977; Genco et al, 1986; Hart, 1996).

The association of *Actinobacillus actinomycetemcomitans* (*A.a.*) with some forms of AP is well documented (Slots, 1976; Mandell and Socransky, 1981; Kornman and Robertson, 1985; Moore et al, 1985; Zambon, 1985; Delaney and Kornman, 1987; Slots et al, 1980). *Porphyromonas gingivalis* (*P.g.*) may also be correlated with periodontal disease in a subset of AP patients (Barrington et al, 2000; Ishikawa et al, 2002). *Prevotella intermedia* (*P.i.*), along with *P.g.* and *Campylobacter rectus*, has also been associated with several forms of AP (Albandar et al, 1997b), and *P.i.* has been found in generalised AP in a Korean population (Lee et al, 2003). In addition, an earlier study associated the LJP form of AP with high prevalence and levels of *P.g.* and *P.i.*, as opposed to *A.a.* (Kamma et al, 1998; Lopez et al, 1996). The prevalence and levels of *P.g.* and *P.i.* were higher in AP (EOP) patients compared with healthy patients, and these bacteria were associated with the generalised and/or rapidly progressing forms of the disease (Albandar et al, 1997b).

Gram-negative periodontal pathogens associated with AP contain lipopolysaccharide (LPS). LPS is capable of activating innate host immunity via activation of neutrophils and monocytes, which phagocytise and thus eliminate the bacteria. In recent years, studies have shown that LPS heterogeneity among pathogenic bacteria may be responsible for altered host immune responses to them, which can result in increased bacterial survival and pathogenicity (Dixon et al, 2005). There may also be genetic predisposition to susceptibility to AP, including genetic control of host immune responses to A.a (Honma et al, 1987). AP appears to be more prevalent in Black than in Caucasian or Hispanic populations (Albandar et al, 1997a).

In this study, we describe a 16-year-old Black female whose chief complaint was gingival pain and tooth mobility. The radiographic exam revealed extensive generalised bone destruction reaching the apices of most teeth. This report describes clinical and laboratory blood test findings, microbiological plaque analysis, salivary analysis, in vitro studies of the patient's neutrophils and monocytes, and the neutrophil priming ability of *P.i.* LPS.

## CASE DESCRIPTION AND RESULTS

The patient was a 16-year-old Black female who presented to the graduate Periodontology Clinic at the University of Tennessee College of Dentistry with chief complaints of gingival pain and tooth mobility. The medical history revealed chronic noncyclic neutropaenia and recurrent otitis media. A general physical examination prior to her presentation at the College of Dentistry was deemed unremarkable except for the severe periodontitis, gingival enlargement, and gingival erythema. There was no lymphandenopathy and no hepatosplenomegaly. The patient was taking no medication. Examination of the mother and two siblings revealed no periodontal pathology.

### Clinical and Radiographic Dental Examinations

Clinical oral examination of the patient revealed severe gingival inflammation and resulting enlargement, heavy accumulation of plaque and heavy calculus formation (Figs 1 and 2). There was generalised type III tooth mobility (Armitage, 2004), probing depths ranging from 8 to 10+ mm on most teeth, and bleeding and plaque scores of 100%.

A full-mouth series of periapical radiographs revealed extreme, generalised bone destruction reaching the apices of most teeth, with a scalloped-like appearance of the alveolar crest. Maxillary left and mandibular right quadrants are shown in Fig 3 and Fig 4 respectively. Routine histological examination of the gingival tissue (not shown) revealed only severe chronic hyperplastic gingivitis and periodontitis.

### Laboratory Blood Test Findings

Laboratory blood test findings revealed neutropaenia (615 cells/µl; normal range 1800–8000 cells/µl), and monocytosis (1968 cells/µl; normal range 200–1100 cells/µl). Complete blood count (CBC) revealed that the numbers of platelets, eosinophils, basophils and lymphocytes were within normal ranges. Prothrombin time and coagulation tests were normal. A lipid panel revealed normal levels of triglycerides and normal total lipoprotein, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol levels. The comprehensive metabolic panel revealed slightly elevated total protein (8.8 g/dl; normal range 6–8.5 g/dl) and globulin (4.9 g/dl; normal range 0.2–4.2 g/dl).

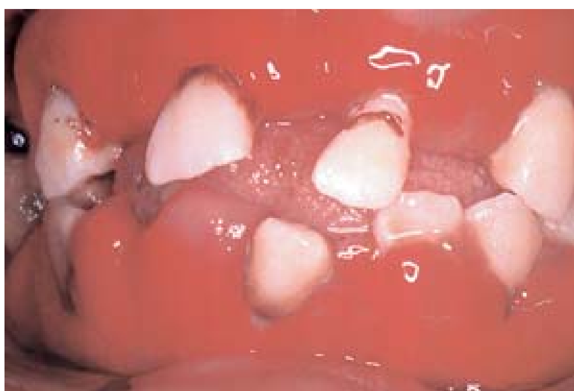

**Fig 1** Intraoral photograph of the facial aspect of the maxillary anterior region at the time of initial clinical examination, showing extreme gingival inflammation, erythema, and enlargement.

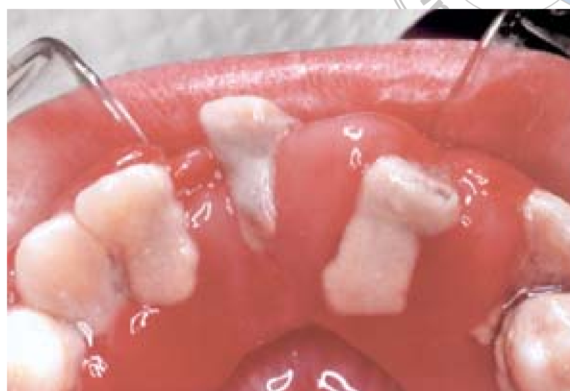

**Fig 2** Intraoral photograph of lingual aspect of anterior mandibular teeth, showing heavy plaque and calculus accumulation, and erythema and enlargement of the gingiva.

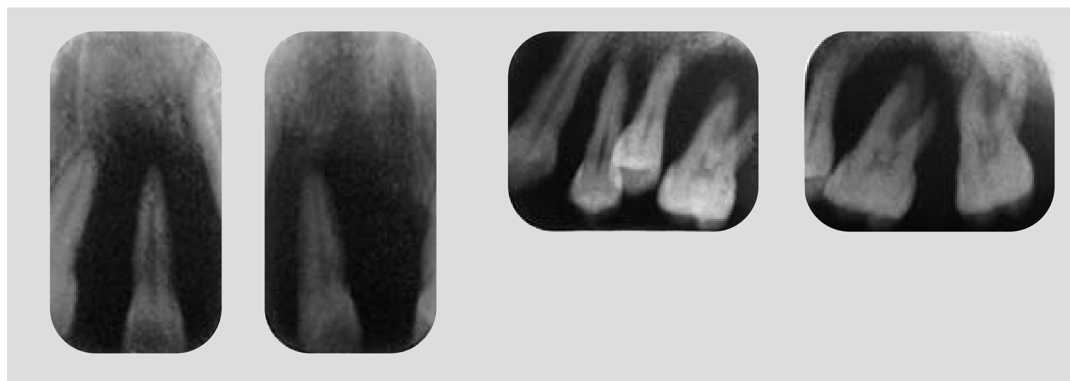

**Fig 3** Periapical radiographs of the maxillary left quadrant at time of initial examination.

## Microbiological Plaque Analysis

Subgingival plaque was collected from sites in each quadrant and analysed by the Microbiological Testing Laboratory at the University of Pennsylvania School of Dental Medicine (Philadelphia, PA). The following bacteria (expressed as % of cultivatable microbiota) were cultured from these samples by growing them in pre-reduced chopped meat glucose broth (Becton, Dickinson and Co.; Franklin Lakes, NJ) and identified by polymerase chain reaction (PCR): *P.i.* (13–48%; normal range 0–2.5%), *Campylobacter* (*Wollinella*) *rectus* (0.036%; normal range 0–2.0%), *Fusobacterium* spp (2%; normal range 0–5.0%). Normal ranges of each species were determined by the results of many analyses at the Microbiological Testing Laboratory

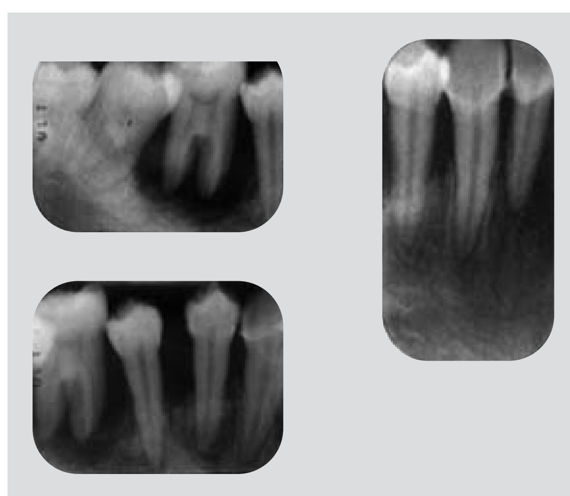

**Fig 4** Periapical radiographs of the mandibular right quadrant at time of initial examination.

at the University of Pennsylvania School of Dental Medicine (personal communication with Dr. Chern H. Lai, Clinical Professor of Periodontics, University of Pennsylvania School of Dental Medicine; 02/20/06). Levels of *P.g.* (5–15%; normal range 0–0.5%) and *Tannerella forsythensis* (1.6–2.5%; normal range 0–1.0%) were expressed as % of microscopic counts as determined by immunofluorescence. Normal ranges of *P.g.* and *T. forsythensis* were determined in unpublished studies (Lai, C.-H.), and in other studies by Lai et al (1987, 1992) (personal communication with Dr. C.-H. Lai; 02/20/06). Morphological analysis revealed spirochaetes (0–1%; normal range 0–5.0%), motile rods (8–18%; normal range 0–5.0%), non-motile rods (6–16%; no defined normal range), and coccoid cells (65–86%; no defined normal range) (Listgarten and Hellden, 1978). Cultivatable *A.a.* was not present in any of the samples.

## IN VITRO STUDIES

### Neutrophil and Monocyte Production of Superoxide and Interleukin-1β (IL-1β)

Neutrophils and mononuclear cells (monocytes and lymphocytes) were isolated from the patient's blood and from blood of a healthy individual who did not have periodontal disease, using a histopaque gradient (Tipton et al, 1990). The number of patient's neutrophils was decreased, compared with the normal subject ($3 \times 10^6$ cells/ml compared with $4.5 \times 10^6$ cells/ml), whereas the number of mononuclear cells was increased, compared with the normal subject ($3 \times 10^6$ cells/ml compared with $2 \times 10^6$ cells/ml). These patterns were consistent with the earlier CBC (see above), but in this determination the patient's neutrophils ($3 \times 10^6$ cells/ml) were within the normal range ($1.8 \times 10^6$ cells/ml to $8 \times 10^6$ cells/ml).

Table 1 shows that the patient's neutrophils produced superoxide normally in response to triggering by 1 µM *N*-formylmethionyl-leucyl-phenylalanine (fMLP), using the cytochrome *c* assay (Johnston et al, 1978). The production of superoxide was normal, both before and after priming the neutrophils with 2 ng/ml *Eschericia coli* LPS. *E. coli* LPS is commonly used for determining the effects of bacterial LPS on cells. Findings were

**Table 1** Effect of LPS on superoxide production by neutrophils from a normal subject and an AP patient. All values are nmol superoxide / million cells. Neutrophils were isolated from peripheral blood and exposed to 2 ng/ml *E. coli* LPS. Superoxide production was measured by fMLP-triggered cytochrome *c* reduction assay

| Treatment | Normal subject | AP patient |
|---|---|---|
| Control | 1.9 ± 0.3 | 3.7 ± 0.2 |
| LPS | 46 ± 3[a] | 33 ± 1[a] |

*[a] Significant priming by LPS ($p < 0.01$ by ANOVA) in both the normal subject and AP patient. There was no significant difference in neutrophil superoxide production between the normal subject and AP patient, with or without LPS.*

similar for the monocytes from the normal and the AP patient (not shown). In both types of cells from the AP patient, the superoxide production in the absence of LPS was low and similar to the normal response, suggesting that the patient's neutrophils and monocytes were not abnormally activated. On the other hand, the AP patient's neutrophils and monocytes were capable of being primed normally by *E. coli* LPS. Therefore, there was no detectable defect in the patient's cells with respect to either priming or triggering of superoxide production.

IL-1β in mononuclear cell culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Unstimulated patient and normal subject mononuclear cells did not secrete IL-1β (again indicating that the patient's cells were not abnormally activated), but the patient's mononuclear cells did secrete normal amounts of IL-1β when stimulated with 2 ng/ml *E. coli* LPS (Table 2).

### Priming of Neutrophils by LPS from *P.i.* and other Periodontal Pathogens

Neutrophils were isolated from blood of six healthy individuals who did not have periodontal disease, as described above. LPS was isolated by using the hot phenol method (Westphal and Jann, 1965) from the following bacteria, obtained from the American Type Culture Collection (ATCC) (Rockville, MD): *P.i.* (ATCC 49046), *A.a.* (ATCC 43718), P.d. (ATCC 33184), and *Fusobacterium nucleatum* (*F.n.*) (ATCC 10953). Previous work showed that *P.i.* LPS was 30-fold less active than *E. coli* or *A.a.* LPS in priming neutrophils for en-

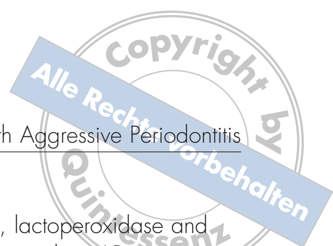


Table 2 Effect of IL-1β production by mononuclear cells from a normal subject and an AP patient. All values are pg of IL-1β per ml (means ± SE, n = 2). Mononuclear cells were isolated from peripheral blood and exposed to 2 ng/ml *E. coli* LPS. IL-1β production was measured by ELISA. There was no significant difference in LPS-stimulated IL-1β production, comparing the normal subject with the AP patient (ANOVA).

| Treatment | Normal subject | AP patient |
|---|---|---|
| Control | 0 | 0 |
| LPS | 27 ± 4 | 31 ± 10 |

Table 3 Salivary myeloperoxidase, lactoperoxidase and total protein levels in normal subjects and an AP patient. Values for normal subjects are reported as means ± SD, n = 20. Myeloperoxidase and lactoperoxidase were measured in expectorated whole mixed saliva by measuring the rate of $H_2O_2$-dependent oxidation of tetramethylbenzidine. Total salivary protein level was determined by the method of Lowry et al (1951).

| | Normal subject | AP patient |
|---|---|---|
| Myelo-peroxidase | 24 ± 14 units/ml | 2654 units/ml |
| Lacto-peroxidase | 6 ± 3 units/ml | 74 units/ml |
| Protein | 2.3 ± 1 mg/ml | 29 mg/ml |

hanced release of fMLP-triggered superoxide release (Aida et al, 1995). In the present study, *P.i.* as well as *P.d.* LPS preparations also had low neutrophil priming activity compared with the other LPS preparations (Fig 5).

### Chemotaxis Assay

To assess their adherence and migration, neutrophils isolated from the patient's blood were labelled with $^{111}$Indium oxine and then incubated with *E. coli* LPS (5 ng/ml) for 15 min. They were assayed for their migration to fMLP in a modified Boyden chamber (Sundqvist and Johansson, 1980). Under these conditions, the patient's PMNs had a normal chemotactic response to fMLP (not shown).

### Salivary Analysis

Myeloperoxidase and human salivary lactoperoxidase were measured in expectorated whole mixed saliva (Thomas et al, 1994) from the patient and from healthy control subjects who did not have periodontal disease. Myeloperoxidase is a marker for neutrophils, which migrate into and through the oral tissues and are shed into the oral cavity. Lactoperoxidase is a marker for secretion of salivary protein. Peroxidase activity was calculated from the rate of $H_2O_2$-dependent oxidation of tetramethylbenzidine (Bozeman et al, 1990). The activity was measured with and without the inhibitor dapsone, to distinguish myeloperoxidase and lactoperoxidase activities, the latter being more sensitive to dapsone inhibition (Thomas et al, 1994). Lactoperoxidase and myeloperoxidase activities in the patient's saliva were much higher

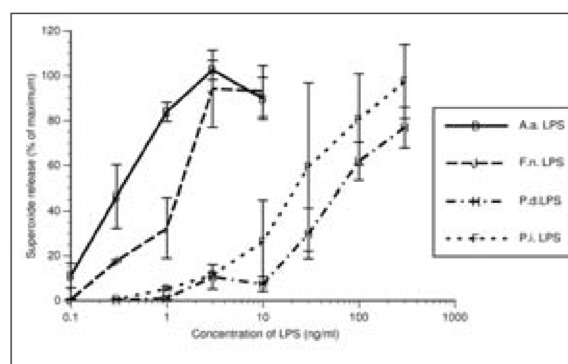


Fig 5 Priming of neutrophils by LPS from four periodontal pathogens for enhanced release of fMLP-triggered superoxide. Values are means ± SE from neutrophils from six different healthy donors. Superoxide release is expressed as % of the maximum superoxide production for each donor.

than in the normal subjects' saliva (Table 3). High myeloperoxidase levels suggest that the patient's neutrophils were able to adhere to oral tissues and migrate into the oral cavity, consistent with normal migration of the neutrophils in response to fMLP (above). The measured lactoperoxidase was high, as was the total salivary protein level (Lowry et al, 1951), compared with control (Table 3).

## DISCUSSION

The definition of AP includes distinctive forms of periodontitis affecting individuals who usually are otherwise healthy (Barrington et al, 2000). The

16-year-old patient presented in this report had a generalised form of AP, which usually affects people under 30 years of age, and which usually manifests as generalised interproximal loss of attachment affecting at least three permanent teeth other than the first molars and incisors (Barrington et al, 2000) (see Figs 3 and 4). AP tends to have a familial distribution, but this patient reported no other family members with AP, and examination of the mother and two siblings revealed no periodontal pathology. The patient in this report was Black, and other reports [in which AP is referred to as juvenile periodontitis (JP)] have shown that the prevalence of AP is greater in Black populations than in Caucasians (Burmeister et al, 1984; Löe and Brown, 1991; Melvin et al, 1991). In another study (in which AP is referred to as EOP), approximately 10% of Black, 5.5% of Hispanic and 1.3% of Caucasian adolescents were shown to have AP (Albandar et al, 1997a).

The patient in the present study was neutropaenic (defined as having a neutrophil level below $1800/mm^3$ in adults), was diagnosed with a non-cyclic form of neutropaenia, and had suffered from recurrent otitis media, which can be associated with neutropaenia (Conway et al, 1987). The neutropaenia could be related to the severe periodontal infection; neutropaenia and monocytosis (which the patent also exhibited) are commonly seen in severe infections. Periodontal therapy, resulting in reduced bacterial load, can restore normal levels of circulating neutrophils in an individual with congenital neutropaenia (Goultschin et al, 2000). In vitro tests of the patient's neutrophils and monocytes showed that they produced superoxide normally in response to fMLP; therefore the patient did not have chronic granulomatous disease, which is characterised by failure of white blood cells to produce activated $O_2$ species, such as superoxide. The patient's neutrophils had a normal chemotactic response to fMLP. However, other reports [in which AP is referred to as localised juvenile periodontitis (LJP)] have indicated that AP patients can have defects in neutrophil chemotaxis (Cianciola et al, 1977; Clark et al, 1977), and AP neutrophils were reported to have fewer receptors for fMLP and other chemotaxis-inducing factors (Van Dyke et al, 1981; Van Dyke et al, 1983). The patient's monocytes secreted normal amounts of IL-1β in response to LPS, and the neutrophils and monocytes had low or non-detectable production of IL-1β in the absence of stimulation with LPS. This indicates that these cells were not abnormally activated. Taken together, these studies suggest that while they were decreased in numbers, the patient's neutrophils and monocytes behaved normally in the absence or presence of stimulatory factors.

The high salivary myeloperoxidase level seen in this patient is consistent with severe inflammation of the oral tissues. This result also suggests that neutrophil adherence and chemotaxis were not compromised in this patient, and supports our in vitro analyses of the patient's neutrophil functions. Statistical analysis was not performed since there was only one AP patient, but the myeloperoxidase value of the AP patient fell outside the range of normal control values and was elevated 100-fold above the control mean. The measured lactoperoxidase was also high, but this result is probably not significant. When the myeloperoxidase activity is very high, it is difficult to measure accurately the small amount of additional peroxidase activity contributed by lactoperoxidase. High salivary protein levels could be due to high numbers of bacteria, leukocytes and other materials shed from oral surfaces into the saliva.

Microbiological analysis of this patient's plaque samples revealed no cultivatable *A.a.*, one of three species of bacteria (including *P.g.* and *T. forsythensis*) that have been described as aetiological factors in the pathogenesis of periodontitis (Ishikawa et al, 2002). *A.a.* has been identified as an important aetiological factor in the form of AP previously referred to as LJP (Zambon et al, 1983; Asikainen, 1986; Mandell et al, 1987). Although there was no *A.a.* in this patient's plaque samples, there were elevated levels of *P.g.* and *T. forsythensis*, and a normal level of *C. rectus*. This is consistent with findings in a report of three other case studies of younger AP patients (Ishikawa et al, 2002), in which it was proposed that bacterial species exist in communities, in these cases including *P.g.* and *T. forsythensis*, but not *A.a.*

*P.i.* has also been associated with generalised AP in a Korean population (Lee et al, 2003), and *P.i.*, along with *P.g.* and *C. rectus*, have been associated with several forms of AP (Albandar et al, 1997b). In addition, an earlier study associated the LJP form of AP with high prevalence and levels of *P.g.* and *P.i.*, as opposed to *A.a.* (Lopez et al, 1996). The prevalence and levels of *P.g.* and *P.i.* were higher in AP (EOP) patients compared with

healthy patients, and these bacteria were associated with the generalised and/or rapidly progressing forms of the disease (Albandar et al, 1997b). Another study described a case of AP (prepubertal) associated with elevated *P.i.* and *P.g.* in the subgingival microflora, but in contrast to our findings, their patient had detectable levels of *A.a.* (Kamma et al, 1998).

The extremely high (~50%) levels of *P.i.*, as well as elevated levels of *P.g.*, in the patient's plaque samples in the present study are consistent with these other reports associating AP with *P.i.* (and *P.g.*). However, the factors contributing to the ability of *P.i.* to establish itself and achieve these levels of predominance in plaque samples from this patient are unknown. *P.i.* produces a wide variety of virulence factors (Slots and Genco, 1984). It secretes a number of enzymes, including collagenases and gelatinase, which degrade structural proteins and promote periodontal breakdown (Slots and Genco, 1984; Maeda et al, 1998; Tatami, 1992). In addition, *P.i.* produces proteases that degrade IgG, IgM, and IgA, which could impair the humoral immune response (Jansen et al, 1995). Other proteases secreted by *P.i.* can degrade CD14, a protein expressed on neutrophils and other myeloid cells, and LPS-binding protein, both necessary for the response of inflammatory cells to LPS, thereby further limiting host defenses (Deschner et al, 2003). In previous work (Aida et al, 1995) and in the present study, *Prevotella* LPS had little ability to prime neutrophils for increased release of superoxide, compared with *E. coli*, *A.a.*, or *F.n.* LPS. The inability of *Prevotella* LPS to significantly prime neutrophils suggests an unusual 'stealth' nature of this LPS. This might be another important virulence factor of *Prevotella*, contributing to its ability to escape efficient detection and elimination by the host immune system. Although the reasons for its development are unknown, this extensive *P.i.* infection and its associated virulence factors, coupled with low numbers of normally functioning circulating neutrophils, may have contributed to the development of AP in this patient.

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