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Short communication

Differential cytokine induction by two types of *Porphyromonas gingivalis*

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The presence of *Porphyromonas gingivalis* with type II *fimA* is strongly associated with adult periodontitis. However, the importance of specific *fimA* types in the immune response is unknown. Two types of *P. gingivalis* (type I and type II) and *Actinomyces naeslundii* were assessed for their degree of cytokine induction in the macrophage-like human cell line U937. Real-time reverse transcriptase polymerase chain reaction was used to determine mRNA expression of 12 cytokines. Significant levels of interleukin (IL)-8 induction and a similar cytokine expression pattern were observed at 6 h postinfection for all three bacterial strains. However, type II *P. gingivalis* infection showed statistically higher levels of IL-1β, IL-8, IL-12 and tumor necrosis factor-α mRNA induction than those of control at 24 h postinfection, whereas type I *P. gingivalis* and *A. naeslundii* showed no significant induction of these cytokines. These data suggest that compared with *A. naeslundii* and type I *P. gingivalis*, type II *P. gingivalis* prolongs the cytokine response. Although other factors may also be involved, the sustained cytokine response induced by type II *P. gingivalis* may play an important role in enhanced periodontal tissue inflammation and destruction.

Key words: cytokine; *fimA* type; *Porphyromonas gingivalis*; real-time polymerase chain reaction

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Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been identified as a major etiologic agent in the pathogenesis of adult periodontitis in humans (4, 15, 18, 26). *P. gingivalis* produces many cell components and macromolecules that have been proposed to function as virulence factors. These factors include lipopolysaccharide, the outer membrane, fimbriae, and numerous end products of metabolism (8, 9, 12, 16). Fimbriae are involved in the interaction of bacteria with other bacterial species and with both soft and hard host tissues. Fimbriae of *P. gingivalis* are filamentous components on the bacterial cell surface, and their subunit protein, fimbrillin (Fim A), is classified into five types on the basis of their nucleotide sequences (19, 20). Most patients with periodontitis harbor type II *P. gingivalis* (Pg-II). In contrast, most healthy subjects carry type I *P. gingivalis* (Pg-I) (2, 3). The virulence factors of Pg-II that cause periodontitis, such as fimbrillin proteins, Arg-specific protease and immunologic properties (1, 6, 7, 13, 23, 28, 29), have been studied extensively, but definitive causes have yet to be identified. Several studies have shown that fimbriae of *P. gingivalis* are able to induce the production of cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α from gingival fibroblasts, epithelial cells and macrophages (10, 11, 23). These cytokines are thought to be important immunologic mediators in periodontal inflammation and the destruction of periodontal tissue. Among the various cells, macrophages play a central role in immune response against invading pathogens. In this study, we evaluated the cytokine response of the macrophage-like human cell line U937 to infection with two types of *P. gingivalis* and *Actinomyces naeslundii*.

P. gingivalis strains 381 (*fimA* type I; Pg-I) and ATCC 49417 (A7A2-10, *fimA* type II; Pg-II) were grown in GAM broth (Nissui, Tokyo, Japan) anaerobically (80% N_2,10% H_2, 10% CO_2) at 37°C. *A. naeslundii* (ATCC12104; An) was cultured in BHI broth (Difco Laboratories, MI).

The U937 cell line was maintained in RPMI1640 medium supplemented with heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 μg/ml) and L-glutamine (2 mM) in 75 cm^2 tissue culture flasks at 37°C in 5% CO_2 in air high humidity. Logarithmic phase cultures at 1×10^6 cells/ml were incubated with 10 nM phorbol myristate acetate (PMA, Sigma, St. Louis, MO) to differentiate into macrophage-like cells for 48 h.

After incubation with PMA, cells were made quiescent for 24 h by incubation in PMA-free fresh medium before experiments.

Bacteria were grown to log-phase cultures. The bacterial cells were washed three times with phosphate-buffered saline and added to U937 cells at approximately 10 : 1 multiplicity of infection in RPMI1640 complete medium without antibiotics.

Total RNA was isolated from cells using the RNeasy total RNA isolation kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. RNA (3 μg per reaction) was reverse-transcribed using a First-Strand kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) at 42°C for 60 min. Following cDNA synthesis, 1 μl of cDNA template was used for each polymerase chain reaction (PCR). Real-time PCR was conducted using Cytokine Gene Expression Plate 1 (Applied Biosystems, Foster City, CA). PCR amplifications for the target cytokine and internal control 18S rRNA were performed in a single well of a capped 96-well optical plate. The reaction conditions were as follows: 5 min at 50°C (one cycle), 10 min at 96°C (one cycle), and 15 s at 95°C and 1 min at 60°C (40 cycles). Gene-specific PCR products were continuously measured by means of ABI PRISM 7700 detection system (Applied Biosystems). Samples were normalized using an internal control, and results are expressed as relative fold increase of the infected over the control (PMA-treated cells), which was used as a calibrator. Results represent the mean value of five independent experiments, and statistical analyses were performed with the Mann-Whitney *U*-test. Differences of $P < 0.01$ were considered to be statistically significant.

IL-1α, IL-1β, IL-4, IL-5, IL-8, IL-10, IL-12p35 (IL-12), IL-12p40, IL-15, interferon (IFN)-γ and TNF-α gene inductions in differentiated U937 cells exposed to three different bacteria (An, Pg-I, Pg-II) were examined by real-time reverse transcription (RT)-PCR. IL-1α, IL-1β, IL-8, IL-10, IL-12 and TNF-α were detectable ($C_T < 36$). Significant levels of IL-8 induction and a similar gene expression pattern were observed at 6 h postinfection for all three bacterial strains (Figs. 1–3). No significant levels of IL-1β and IL-12 could be detected at 6 h postinfection. Pg-II showed statistically higher levels of IL-1β, IL-8, IL-12 and TNF-α induction than those of controls at 24 h postinfection, whereas Pg-I and An showed no significant inductions of these cytokines. All three bacterial strains produced statistically higher levels of IL-1α and IL-10 expression at 24 h postinfection.

Recent studies provide clinical as well as biochemical evidence that FimA clonal variation may contribute to the periodontopathogenicity of *P. gingivalis*. It has been shown that Pg-II adheres to and invades epithelial cells significantly more than

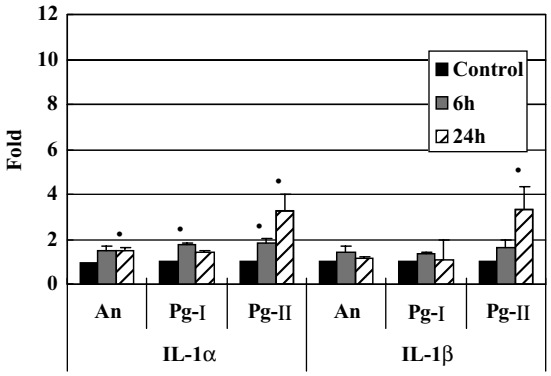

Fig. 1. IL-1α and IL-1β mRNA expression by infected U937 cells. PMA-treated U937 cells were infected for 6 h and 24 h with *A. naeslundii* (An), type I *P. gingivalis* (Pg-I), and type II *P. gingivalis* (Pg-II). Total RNA was extracted and cytokine mRNAs were determined by real-time RT-PCR. Results are mean fold increases (+ SEM) in cytokines for five separate experiments. Each experiment was run in duplicate. $^*P < 0.01$.

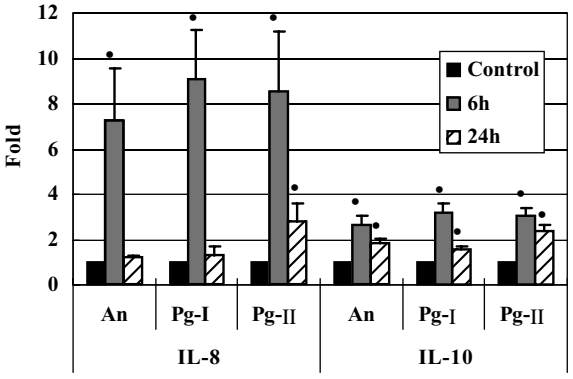

Fig. 2. IL-8 and IL-10 mRNA expression by infected U937 cells. PMA-treated U937 cells were infected for 6 h and 24 h with *A. naeslundii* (An), type I *P. gingivalis* (Pg-I) and type II *P. gingivalis* (Pg-II). Total RNA was extracted and cytokine mRNAs were determined by real-time RT-PCR. Results are mean fold increases (+SEM) in cytokines for five separate experiments. Each experiment was run in duplicate. $^*P < 0.01$.

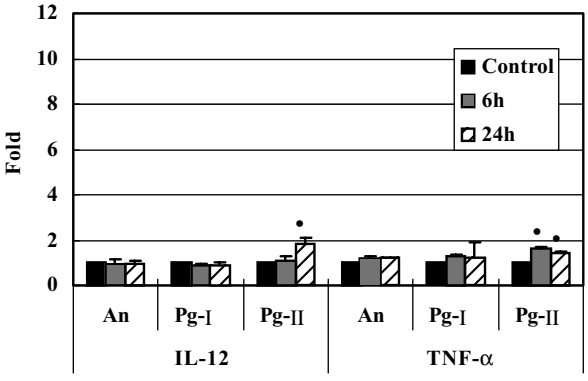

Fig. 3. IL-12 and TNFα mRNA expression by infected U937 cells. PMA-treated U937 cells were infected for 6 h and 24 h with *A. naeslundii* (An), type I *P. gingivalis* (Pg-I) and type II *P. gingivalis* (Pg-II). Total RNA was extracted and cytokine mRNAs were determined by real-time RT-PCR. Results are mean fold increases (+SEM) in cytokines for five separate experiments. Each experiment was run in duplicate. $^*P < 0.01$.

other *P. gingivalis* strains (14, 30). The type of fimbriae also seems to correlate with other properties such as hemagglutination and elastase-like activity (17, 22, 25). Studies using the reference strains *P. gingivalis* ATCC 33277 (Type I), 381 (Type I), W50 (Type IV) and clinical isolates showed differences in their virulence in mice (27). However, little explanation has been given for the differing virulence of *P. gingivalis* with different FimA genotypes. Recent studies showed that FimA interacts with CD14 and CD11/CD18, which serve as coreceptors in a TLR/CD14/CD11/CD18 multireceptor complex. Subsequent Toll-like receptor intracellular signaling leads to NF-κB activation and induction of cytokines (24). A previous study showed that synthetic peptide ALTTE based on the amino acid sequences of the fimbrillin of *P. gingivalis* induced cytokine production in human monocytes (21). On the other hand, the analog synthetic peptide GLTTE, in which an alanine residue was replaced with glycine, did not induce cytokine production. These data suggest that the property of *P. gingivalis* to induce cytokine release may be dependent, at least partially, on its fimbrillin types. Therefore, in further studies, it will be very important to define in more detail the functional differences among FimA variants of *P. gingivalis*.

In conclusion, our results suggest that compared with An and Pg-I, Pg-II prolongs the cytokine response. Although other factors may also be involved, the sustained cytokine response induced by Pg-II may play an important role in enhanced periodontal tissue inflammation and destruction.

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