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Differential effects of five *Aggregatibacter actinomycetemcomitans* strains on gingival epithelial cells

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Introduction: We investigated gingival epithelial cell proliferation and expression of interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM-1) in response to *Aggregatibacter actinomycetemcomitans* serotypes a, b, and c.
Methods: Human gingival cells (Ca9-22) were cultured in bacterial extracts prepared from five strains of *A. actinomycetemcomitans*: ATCC 43717 (serotype a); ATCC 29524, ATCC 29522, and ATCC 43718 (all serotype b); and ATCC 43719 (serotype c).
Results: In bacterial extracts of ATCC 29522, cell growth was significantly impaired, while the expression of IL-8 and ICAM-1 was significantly increased. The level of induction in response to the other strains was minimal.
Conclusion: Our results indicate that the five strains of *A. actinomycetemcomitans* have distinct effects on the abilities of human gingival epithelial cells to proliferate and to produce proinflammatory factors.

Key words: *Aggregatibacter actinomycetemcomitans*; gingival epithelial cells; intercellular adhesion molecule 1; interleukin-8; serotype

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Aggregatibacter actinomycetemcomitans, a gram-negative, capnophilic coccobacillus, is a major pathogen in the initiation and progression of periodontitis (1, 3, 17, 23); however, it has also been sporadically isolated in non-oral infections, including endocarditis, pneumonia, and septicemia (6, 7, 18, 21, 29). *A. actinomycetemcomitans* is classified into six distinct serotypes (a to f) based on the polysaccharides present on the surface of the organism, which function as immunodominant antigens. Of the six known serotypes, a, b, and c are the most prevalent in the oral cavity (1, 12, 15, 19, 28). Serotype b is more frequently observed in patients with aggressive periodontitis than in healthy individuals (1, 2, 10, 22, 27, 28). The predominance of serotype b in subjects with periodontitis suggests a greater periodontopathic potential, but the exact mechanism of pathogenicity is unclear.

Epithelial cells function as a mechanically protective barrier against invasion by pathogenic organisms. Moreover, increasing evidence indicates that gingival epithelial cells play a role in local innate immune responses (16, 24, 25). Epithelial cells express chemokines that attract and activate leukocytes, and they express adhesion molecules that mediate leukocyte migration. The expression of these molecules plays an important role in the initial stages of inflammation. Interleukin-8 (IL-8), a neutrophil chemoattractant and activator, is induced in gingival epithelial cells by several periodontopathic bacteria (4), while intercellular adhesion molecule 1 (ICAM-1) is the ligand for lymphocyte function-associated antigen-1 expression in leukocytes (9). In human gingival epithelium, as these molecules are expressed, a gradient is formed with the highest level on the epithelial cells facing the tooth surface. These gradients play an essential role in directing the migration and activation of leukocytes (8). Previous studies have shown that IL-8 and ICAM-1 are upregulated in gingival epithelial cells following challenge with *A. actinomycetemcomitans* (13, 14). The present study was undertaken to investigate the growth inhibition and the regulation of IL-8 and ICAM-1 in gingival epithelial cells in response to challenge with three serotypes of *A. actinomycetemcomitans*.

Materials and methods

Cell culture

The human gingival carcinoma cell line Ca9-22 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in minimum essential medium (Asahi Technoglass, Tokyo, Japan) containing 10% fetal bovine serum (Asahi Technoglass) and 1% penicillin–streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Bacterial extracts

A. actinomycetemcomitans strains ATCC 43717 (serotype a); ATCC 29524, ATCC 29522, ATCC 43718 (all serotype b); and ATCC 43719 (serotype c) were grown in trypticase soy broth agar with 0.6% yeast extract at 37°C in an anaerobic chamber (80% N_2, 10% H_2, and 10% CO_2) until an optical density of about 1.0 at 550 nm was reached. The cells were then harvested by centrifugation at 10,000 ***g*** for 15 min at 4°C. Bacterial extracts were prepared by sonication at 20 W for 5 min; the insoluble debris was subsequently removed by centrifugation at 10,000 ***g*** for 30 min at 4°C. The supernatant was then filter-sterilized and stored at −80°C until use. The protein concentration of each extract was determined using the Bradford protein assay (Bio-Rad, Hercules, CA).

Heat inactivation of the bacterial extracts

The extracts were kept at temperatures ranging from 60 to 90°C for 30 min before being added to the cell cultures.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-8 in each culture supernatant was determined by ELISA using commercially available ELISA kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

Cell proliferation

Cell suspensions, quantified using a hemocytometer, were seeded at 1.0×10^5 cells/well into six-well plates (Iwaki, Tokyo, Japan) and cultured with bacterial extracts of different serotypes of *A. actinomycetemcomitans* at 37°C in a humidified environment of 5% CO_2. The data are expressed as the mean ± standard deviation (SD) of five independent experiments. Each experiment was performed in triplicate.

Real-time polymerase chain reaction (PCR)

Cells were seeded at 1.0×10^5 cells/well in six-well plates. After 96 h of incubation, the medium was replaced with either fresh medium containing bacterial extract or fresh medium alone (control) and the cells were grown for an additional 6 h at 37°C in a humidified atmosphere of 5% CO_2. After treatment, the cells were harvested using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and resuspended in phosphate-buffered saline (PBS).

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using a Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ). The primer and probe sets for ICAM-1 and IL-8 were obtained from Applied Biosystems (Tokyo, Japan). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the following parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. The expression level of each gene was first normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample, and the relative differences between the control and treatment groups were calculated and expressed as relative increases, with the control set at 100%. The data are shown as the mean ± SD of six independent experiments. Each experiment was run in duplicate.

Flow cytometry

Cells were seeded at 1.0×10^5 cells/well in six-well plates as described above. After 96 h of incubation, the medium was replaced with either fresh medium containing bacterial extract or fresh medium alone (control) and the cells were incubated for an additional 24 h at 37°C in a humidified atmosphere of 5% CO_2. After treatment, the cells were harvested using 0.25% trypsin/EDTA and resuspended in PBS (pH 7.4). The cells were then incubated with antihuman ICAM-1 monoclonal antibody (fluorescein isothiocyanate-conjugated CD54; Beckman Coulter, Fullerton, CA) at 20 µl of antibody/10^6 cells. The suspension was incubated with gentle shaking for 30 min at 4°C in the dark. After each step, the cells were washed and resuspended in PBS. Flow cytometry was performed using EXPO32 (Beckman Coulter). The data shown are representative of three independent experiments.

Statistical analysis

Data were analyzed for significance using a one-way analysis of variance with Bonferroni post-test correction for multiple comparisons. Student's *t*-test was used to determine the statistical significance of differences between control and test group.

Results

The effect of *A. actinomycetemcomitans* on proliferation of Ca9-22 cells was investigated by culturing the cells in bacterial extracts prepared from five different strains. Following cultivation in the extracts of four strains, the cells' growth in each culture was reduced in comparison to the control (Fig. 1A). In contrast, no significant reduction was observed for Ca9-22 cells grown with ATCC 43717. Cell growth was significantly impaired in bacterial extracts of ATCC 29522 at 6 h (Fig. 1B).

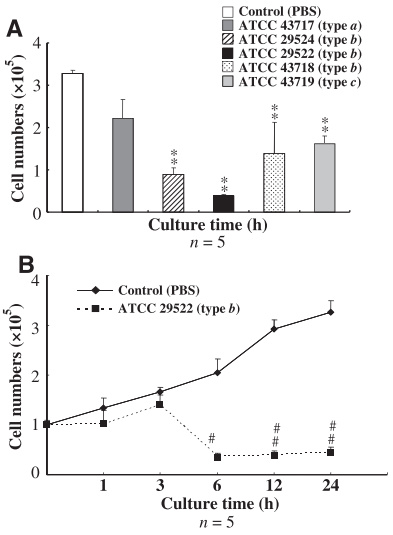

Fig. 1. Effects of the bacterial extracts on cell proliferation. Ca9-22 cells (1.0×10^5 cells/well) were cultured in 2-ml aliquots of medium that contained different serotypes of bacterial extracts (3 µg/ml) at 37°C in a humidified atmosphere of 5% CO_2. Cell numbers were assessed at 24 h (A) The data are expressed as the mean ± standard deviation (SD) of five independent experiments. Each experiment was performed in triplicate. $^{**}P < 0.01$. (B) Cell numbers were also assessed using ATCC 29522 at each time-points; ## $P < 0.01$.

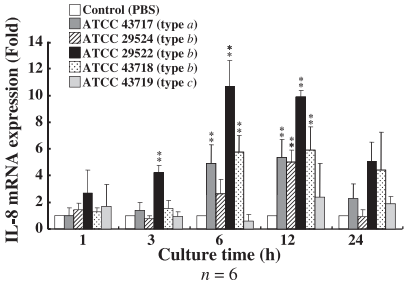

Fig. 2. Effects of the bacterial extracts on interleukin-8 (IL-8) messenger RNA (mRNA) expression. Ca9-22 cells were incubated with medium that contained bacterial extracts (3 μg/ml) for 1, 3, 6, 12, and 24 h. Real-time polymerase chain reaction was used to monitor the levels of IL-8 mRNA which were normalized to that of GAPDH; the relative differences between the control and experimental groups are expressed as relative increases, with the control set at 100%. The data are shown as the mean ± SD of six independent experiments. Each experiment was run in duplicate. $^{**}P < 0.01$.

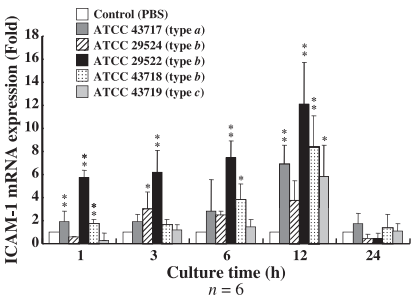

Fig. 4. Effects of the bacterial extracts on intercellular adhesion molecule 1 (ICAM-1) messenger RNA (mRNA) expression. Ca9-22 cells were incubated with medium that contained bacterial extracts (3 μg/ml) for 1, 3, 6, 12, and 24 h. Real-time polymerase chain reaction was used to monitor the levels of ICAM-1 mRNA which were normalized to that of GAPDH; the relative differences between the control and experimental groups are expressed as relative increases, with the control set at 100%. The data are shown as the mean ± SD of six independent experiments. Each experiment was run in duplicate. $^{**}P < 0.01$; $^{*}P < 0.05$.

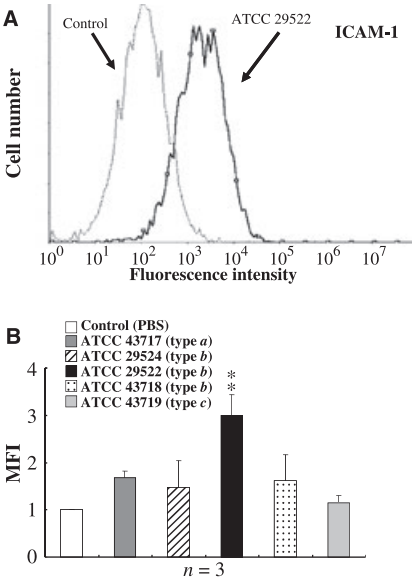

Fig. 5. Effects of the bacterial extracts on the levels of intercellular adhesion molecule 1 (ICAM-1). Cells were incubated with medium that contained bacterial extracts (3 μg/ml) for 24 h. Flow cytometry was used to monitor the levels of ICAM-1 (A) The solid line and dotted line indicate the bacterial extract of *Aggregatibacter actinomycetemcomitans* ATCC 29522 and the control, respectively. (B) Mean fluorescence intensities (MFI) of ICAM-1 expression were assessed by flow cytometry. The data are shown as the mean ± SD of three independent experiments. Each experiment was run in duplicate. $^{*}P < 0.05$.

The induction of IL-8 in Ca9-22 cells exposed to five different strains of *A. actinomycetemcomitans* was examined by real-time PCR. Significant induction was observed 6 h postinfection with four of the strains (Fig. 2). ATCC 29522 induced a significantly higher level of IL-8 expression than the control at 3, 6, and 12 h poststimulation, whereas the level of IL-8 expression by cells cultured in ATCC 43719 was not significant. IL-8 secretion significantly increased following stimulation with the ATCC 29522 extract. In contrast, only minimal IL-8 secretion was observed in response to the other four strains (Fig. 3).

We next investigated the effect of the bacterial extracts on adhesion molecule expression in Ca9-22 cells by real-time PCR. ICAM-1 expression was significantly increased 12 h poststimulation by all of the strains except ATCC 29524 (Fig. 4). As shown by flow cytometry (Fig. 5A,B), ICAM-1 expression increased significantly following stimulation with the ATCC 29522 extract; however, minimal induction of ICAM-1 was observed in response to the other four strains.

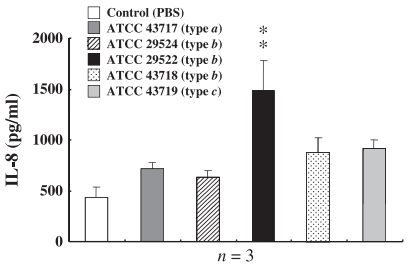

Fig. 3. Effects of the bacterial extracts on interleukin-8 (IL-8) secretion. Cells were incubated with medium that contained bacterial extracts (3 μg/ml) for 24 h. Culture supernatants were assayed for IL-8 using enzyme-linked immunosorbent assay kits. The data are shown as the mean ± SD of six independent experiments. Each experiment was run in duplicate. $^{*}P < 0.05$.

Additional experiments performed with heat-modified sonicates of *A. actinomycetemcomitans* indicated that the stimulating factor in the bacterium was a protein. For example, the effects of the sonicates were considerably reduced with increasing temperature (Fig. 6A–C). Similar results were obtained in other strains (data not shown).

Discussion

A. actinomycetemcomitans serotype b produces cytotoxic membrane microvesicles (16), whereas the other known serotypes do not, which may account for the association between serotype b and aggressive periodontitis. Serotype b is the major serotype in patients with aggressive periodontitis (1), while several studies have shown that no obvious difference exists in the distribution of serotypes a, b, and c. These observations suggest that the pathogenic properties of *A. actinomycetemcomitans* serotype b strains may differ. Therefore, we compared the epithelial cell response to challenge with three *A. actinomycetemcomitans* serotype b strains. Significant inhibition of cell growth and enhancement of IL-8 and ICAM-1 expression in response to ATCC 29522 extracts was observed, which suggests that considerable variation exists among these serotype b strains.

Our data also indicate that the components responsible for the inhibition of cell growth and enhanced expression of IL-8 and ICAM-1 are heat-sensitive molecules. Previous studies have shown that *A. actinomycetemcomitans* is able to degrade host tissue proteins (11, 25), and this activity has been identified for both bacterial cellular material and the culture medium (20). Furthermore, protease from *A. actinomycetemcomitans* inhibited the proliferation of human gingival epithelial cells, and the heat-inactivated protease was similar to that in the control. Protease is secreted *in vivo* by *A. actinomycetemcomitans* in the plaque of periodontal pockets, and also during the invasion of periodontal tissues by *A. actinomycetemcomitans* (5). It may function as a major virulence factor in the development of periodontal disease. However, the mechanisms by which

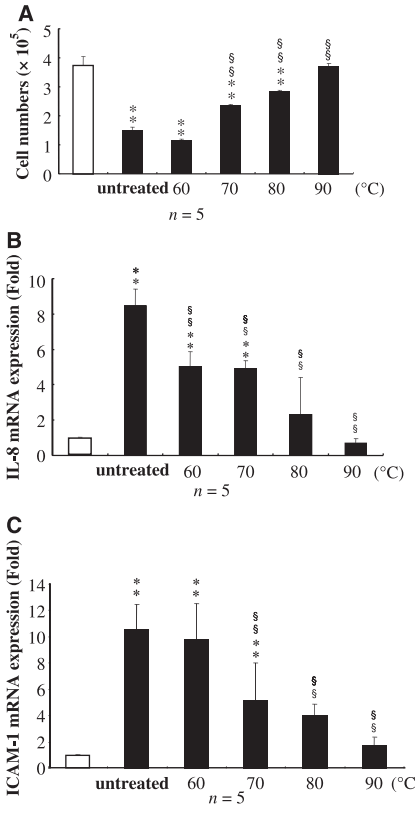

Fig. 6. Heat inactivation of the bacterial extracts. The bacterial extracts were kept at temperatures ranging from 60 to 90°C for 30 min before being added to the cell cultures. (A) Effects of heat-treated *Actinobacillus actinomycetemcomitans* ATCC 29522 bacterial extracts (3 μg/ml) on cell proliferation at 24 h and (B) interleukin-8 messenger RNA (IL-8 mRNA) expression at 6 h were assessed. (C) Intercellular adhesion molecule 1 (ICAM-1) mRNA expression at 12 h was assessed. The data are shown as the mean ± SD of five independent experiments. Each experiment was run in duplicate. $^{**}P < 0.01$ vs. control; §§$P < 0.01$ vs. untreated.

A. actinomycetemcomitans extracts affect gingival epithelial cells require further investigation.

In conclusion, the present study describes the distinct effects of three *A. actinomycetemcomitans* serotype b strains on human gingival epithelial cell proliferation and proinflammatory cytokine production. These effects may produce different host responses that would subsequently influence the development of periodontal lesions. Further study is necessary to clarify the functional differences among the strain variants of *A. actinomycetemcomitans*.

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