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Porphyromonas gingivalis with either *Tannerella forsythia* or *Treponema denticola* induces synergistic IL-6 production by murine macrophage-like J774.1 cells

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ABSTRACT

Background: Chronic periodontitis is caused by mixed bacterial infection. *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* are frequently detected in deep periodontal pockets. We demonstrate that these bacteria induce proinflammatory cytokine production by the mouse macrophage-like cell line J774.1.

Materials and methods: J774.1 cells were incubated with and without bacteria for 24 h in 96-well flat-bottomed plates. The culture supernatants were analyzed by enzyme-linked immunosorbent assay for secreted mouse interleukin (IL)-6, monocyte chemoattractant protein-1, IL-23, IL-1β and tumor necrosis factor-α. The cytokine concentrations were determined using a standard curve prepared for each assay.

Results: Mixed infection with *P. gingivalis* and either *T. forsythia* or *T. denticola* at 10^5 CFU/ml acted synergistically to increase IL-6 production, but not monocyte chemoattractant protein-1, IL-23, IL-1β or tumor necrosis factor-α production. Gingipain inhibitors KYT-1 and KYT-36 inhibited IL-6 production by J774.1 cells incubated with 10^5 CFU/ml of mixed bacteria.

Conclusion: These results suggest that *P. gingivalis* with either *T. forsythia* or *T. denticola* directly induces synergistic IL-6 protein production and that gingipains play a role in this synergistic effect.

1. Introduction

Periodontitis is caused by mixed bacterial infection in the oral cavity. *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* are frequently detected in deep periodontal pockets [1,2]. In addition, periodontopathogens have recently been associated with various systemic diseases, such as arteriosclerosis, pre-term birth, and respiratory infections. Poor oral conditions that cause periodontal diseases are associated with pneumonia. In fact, bacteria found in aspiration pneumonia and lung abscesses include periodontal pathogenic bacteria [3–5]. *P. gingivalis*, an anaerobic periodontopathogen, contains lipopolysaccharides (LPS), fimbriae, lipoproteins, and proteinases, including gingipains. These components increase the production of inflammatory cytokines and chemokines in human and mouse cells [6–8]. *T. forsythia* has been found in subgingival plaques in patients with severe periodontitis along with *P. gingivalis* [9,10], and cooperates with *P. gingivalis* to form severe abscesses in rabbits and mice [11,12]. This interaction is also seen *in vitro*. Sonicated cell extracts from *T. forsythia* stimulate the growth of *P. gingivalis* [13], while the outer membrane vesicles produced by *P. gingivalis* enhance the attachment to and invasion of epithelial cells by *T. forsythia* [14]. Mixed infection with *P. gingivalis* and *T. denticola* induces experimental pneumonia in mice [15].

To elucidate the reasons for severe inflammatory responses to mixed infection with *P. gingivalis* and either *T. forsythia* or *T. denticola*, we measured proinflammatory cytokine production by the J774.1 mouse macrophage-like cell line.

2. Materials and methods

2.1. Cells, bacteria and reagents

Mouse macrophage-like J774.1 cells were obtained from the Institute of Development, Aging and Cancer at Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in an incubator with 5% CO_2. *P. gingivalis* ATCC 33277 was grown in GAM broth (Nissui, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). *T. forsythia* ATCC 43037 was grown in brain heart infusion broth (Gibco) supplemented with hemin (5 μg/ml), menadione (1 μg/ml), 5% FBS and 0.001%

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N-acetylmuramic acid (Sigma). *T. denticola* ATCC 35405 was grown in trypticase-yeast extract-gelatin-volatile fatty acid-serum broth (TYGVS) supplemented with 5% rabbit serum (Equitech-Bio Inc., Kerrville, TX, USA). The bacterial cultures were incubated at 37 °C under anaerobic conditions. For infection experiments, *P. gingivalis* or *T. forsythia* cultures were grown to a concentration of 10^8 cells/ml as determined by an optical density of 0.8 at 600 nm. *T. denticola* cells were counted using a Petroff-Hausser bacterial chamber (Hausser and Son, Philadelphia, PA, USA). KYT-1 and KYT-36, inhibitors for Arg-gingipain and Lys-gingipain [16], respectively, were purchased from Peptide Institute Inc. (Osaka, Japan).

2.2. Measurement of cytokine production

P. gingivalis, *T. forsythia* and *T. denticola* were centrifuged at 1500 × *g* for 10 min and washed twice with RPMI 1640 medium before cocultivation. J774.1 cells (2×10^5 cells/well) were incubated with and without 10^4–10^6 CFU/ml of single and mixed bacteria in RPMI 1640 medium with 10% FBS at 37 °C for 24 h in 96-well flat-bottomed plates (Falcon, Franklin Lakes, NJ, USA). After incubation, the culture supernatants were centrifuged at 500 × *g* for 5 min and collected for cytokine assays. The culture supernatants were analyzed by ELISA for secreted mouse interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1), IL-23, IL-1β and tumor necrosis factor (TNF)-α (eBioscience, San Diego, CA, USA). The cytokine concentrations were determined by interpolation from a standard curve prepared for each assay. We used a lactate dehydrogenase (LDH) cytotoxicity assay to confirm that incubation with the mixed bacteria did not affect J774.1 cell viability. The LDH assay was performed according to the manufacturer's instructions (Cytotoxicity Detection Kit; Roche Diagnostics, Rotkreuz, Switzerland). For gingipain inhibition assays, *P. gingivalis* cells were washed twice with RPMI 1640 medium, preincubated with various concentrations of KYT-1 or KYT-36 in serum-free medium at 37 °C for 30 min, and added to J774.1 cells. Next, we added RPMI 1640 medium containing 20% FBS with or without *T. forsythia* or *T. denticola*.

2.3. Statistical analysis

Data were analyzed using a one-way analysis of variance and either the Bonferroni or Dunn method. The results are presented as the means ± SE.

3. Results

3.1. P. gingivalis *with either* T. forsythia *or* T. denticola *synergistically induces IL-6 but not MCP-1, IL-23, IL-1β and TNF-α production by J774.1 cells in mixed infections with a small number of bacterial cells*

We investigated the release of the proinflammatory cytokines IL-6, MCP-1 and IL-23 by J774.1 cells. *P. gingivalis* and *T. forsythia* are each able to induce IL-6, MCP-1 and IL-23 production in a dose-dependent manner (Fig. 1A–C). However, *T. denticola* did not induce cytokine production. Inoculation with 10^4 CFU/ml of single and

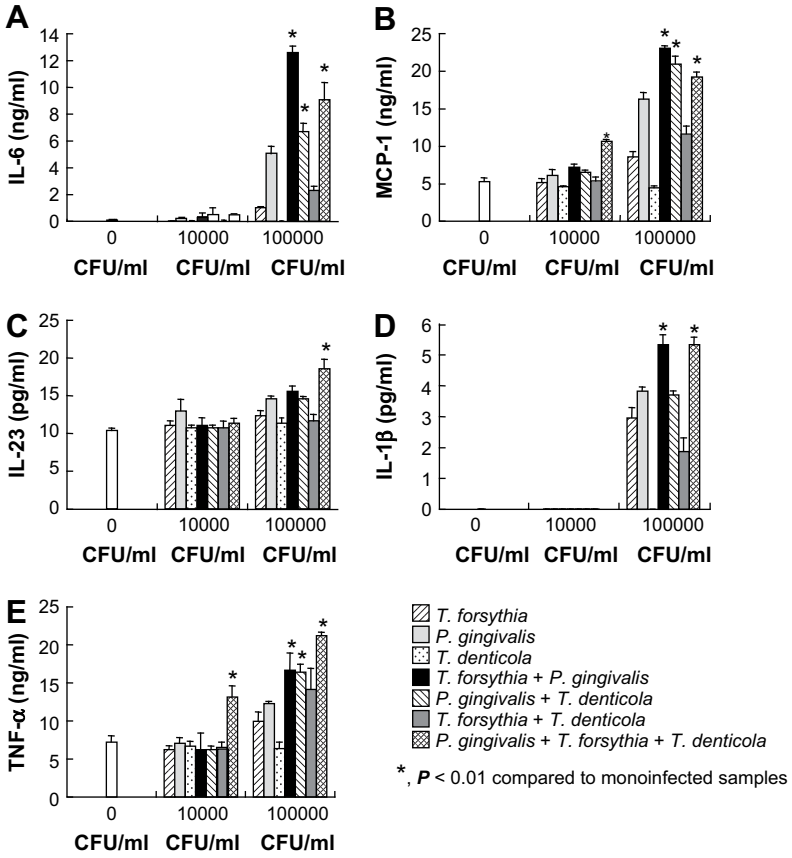

Fig. 1. Secretion of IL-6 (A), MCP-1 (B), IL-23 (C), IL-1β (D) and TNF-α (E) by J774.1 cells following mono- or mixed infections with whole cells of *P. gingivalis*, *T. forsythia* and *T. denticola*. J774.1 cells were co-cultured with or without the indicated concentration of *P. gingivalis* and/or *T. forsythia* and/or *T. denticola* for 24 h. After incubation, cytokine secretion in the supernatants was assessed by ELISA. Each sample was assayed in triplicate. The data are given as the mean ± SE. *$P < 0.01$ compared to the monoinfected samples. The experiments were performed three times.