A 4.1-Kilodalton Polypeptide in the Cultural Supernatant of *Mycoplasma fermentans* Is One of the Substances Responsible for Induction of Interleukin-6 Production by Human Gingival Fibroblasts

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Received 3 April 2001/Returned for modification 20 June 2001/Accepted 20 August 2001

Most bacterial modulins, which are involved in inflammatory responses, are cell wall components. However, mycoplasmas, wall-less microbes, stimulate lymphocytes, natural killer cells, and monocytes/macrophages to produce cytokines and chemokines (17). The cytokine production-inducing activity exists mostly in fractions containing hydrophobic compounds such as lipoproteins or lipoglycans (17). One macrophage-stimulating lipopeptide with an approximate molecular mass of 2 kDa has been extracted and isolated from *Mycoplasma fermentans* cells and is named macrophage-activating lipopeptide 2 (MALP-2) (16). It has been reported that MALP-2 induces production of interleukin-6 (IL-6), IL-8, IL-10, tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein 1, and macrophage inflammatory protein-1 by human monocytes (11). *M. fermentans* and *M. salivarium* induce production of IL-6 and IL-8 by human gingival fibroblasts (HGF) (6, 23) and cell surface expression of intercellular adhesion molecule 1 in HGF (3). The activity of *M. salivarium* exists in cell membrane and intracellular fractions, and a water-soluble active entity (20.6-kDa protein) has been partially purified from intracellular fractions (6). Except for this, water-soluble substances in mycoplasmas capable of activating HGF have not been identified. In this study, we found that cultural supernatants of *M. fermentans* induced cytokine production by HGF and we tried to purify and characterize the active entities.

**Organisms and culture conditions.** *M. fermentans*. ATCC 19989 was grown in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% (vol/vol) horse serum (GIBCO Life Technologies, Inc., Grand Island, N.Y.), 1% (wt/vol) yeast extract (Difco), 1% (wt/vol) D-glucose, 0.002% (wt/vol) phenol red, 0.05% (wt/vol) thallium acetate, and penicillin G (1,000 U/ml). Cultures were incubated at 37°C for 6 h. The culture plate was then centrifuged at 400 × g for 10 min.

*THP-1* (a myelomonocytic cell line, JCRB 0112.1) cells obtained from the Health Science Research Resources Bank (Osaka, Japan) were cultured in RPMI 1640 medium (GIBCO) containing 10% (vol/vol) FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml) in a plastic culture bottle. The cells were collected by centrifugation at 400 × g for 10 min, washed three times with RPMI 1640 medium without FBS [RPMI (−) medium], and suspended in RPMI (−) medium at a cell concentration of 5 × 10^6/ml. A 200-μl volume of the cell suspension was transferred to round-bottom wells of a microculture plate, and a test stimulant in 20 μl of RPMI (−) medium was then added to the wells. Further incubation was done at 37°C for 6 h.

**Assay for cytokines.** The concentrations of IL-6, TNF-α, and IL-1β in the cultural supernatant were determined by using TiterZyme enzyme-linked immunosorbent assay kits (PerSeptive Diagnostics Inc., Cambridge, Mass.) in accordance with the manufacturer’s instructions. The detection limits of the enzyme-linked immunosorbent assay kit are 10.9 pg/ml for IL-6, 28.1 pg/ml for TNF-α, and 2.69 pg/ml for IL-1β.

**Ability of *M. fermentans* cultural supernatants to induce IL-6 production by HGF.** *M. fermentans* was grown in medium identical to that described above. A 2-ml aliquot of the culture...
FIG. 1. Growth of *M. fermentans* in liquid medium and IL-6 production-inducing activity in the cultural supernatant. *M. fermentans* was grown in liquid medium, and a 2-ml aliquot of the culture was taken periodically and divided into two portions. One portion was used to determine the pH (□) and the number of viable cells (CFU/ml, □). The other was centrifuged at 100,000 × g for 1 h to separate the cultural supernatant, which was used to determine the ability to induce IL-6 production by HGF (●).

was taken periodically and divided into two portions. One portion was used to determine the pH and the number of viable cells by using liquid and agar media described by Hayflick (7). The other portion was centrifuged at 100,000 × g for 1 h to separate the cultural supernatant, which was used to determine IL-6 production-inducing activity.

**Determination of amounts of proteins, amino groups, and carbohydrates.** The amount of proteins was determined by the method of Dully and Grieve (4), the amount of carbohydrates by the phenol-sulfuric acid method (5).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 16% polyacrylamide gels by the method of Laemmli (13). Proteins were stained by using a silver stain plus kit (Bio-Rad Laboratories, Hercules, Calif.). Kaleidoscope polypeptide standards (Bio-Rad) were used to estimate molecular weights.

**Enzyme treatment.** Proteinase K was purchased from Takara Biomedicals (Otsu, Japan), and lipoprotein lipase (EC 3.1.1.34) was purchased from Sigma-Aldrich Co. (St. Louis, Mo.). A 0.02:1 (wt/wt) reaction mixture of enzyme and the sample separated from the cultural supernatant of *M. fermentans* was treated at 37°C for 2 h and then tested for IL-6 production-inducing activity.

**Antiserum.** Japanese White rabbits were injected subcutaneously with 2 ml of a 1:1 (vol/vol) mixture of Freund’s incomplete adjuvant (Difco) and a cell suspension (500 μg of protein/ml) of *M. fermentans* grown in PPLO broth (Difco) supplemented with 2% (vol/vol) rabbit serum (GIBCO) on day 0, subcutaneously with the same volume of the same mixture on day 14, and intraperitoneally with 1 ml of the cell suspension on day 21. Sera were drawn before immunization (preimmune serum) and 1 week after the final immunization (anti-*M. fermentans* serum).

**IL-6 production-inducing activity in cultural supernatants of *M. fermentans.** Almost in parallel with viable counts, the IL-6 production-inducing activity increased with prolongation of the incubation time up to 150 h, at which point the activity had reached a maximum (Fig. 1). Then, the activity decreased gradually, to approximately 80% of the maximum at 220 h, and remained at that level for up to 380 h. Activity was not detected in medium not inoculated with the organism.

**Purification of active entities.** Proteins in the cultural supernatant were precipitated with ammonium sulfate between 0 and 30%, 30 and 60%, and 60 and 100% saturation and were examined for activity. Most of the activity (97%) was recovered in the proteins precipitating with ammonium sulfate between 30 and 60% saturation (P60, Table 1). In order to characterize the active entities in P60, the effect of enzymes on the activity was investigated. The activity was reduced to 30% by treatment with proteinase K and lipoprotein lipase (Table 2), suggesting that proteins and lipoproteins are involved in expression of the activity.

P60 was fractionated by reversed-phase high-performance liquid chromatography (HPLC) with a preparative Nucleosil 120-7 C18 column (10 by 300 mm; Chemco Scientific Co. Ltd., Osaka, Japan). Fractionation was carried out by using the following program: at time zero, 5% N,N-dimethylformamide (DMF)–95% milli-Q water (MQW); at 60 min, 5% DMF–95% MQW; at 15 min, 5% DMF–95% 2-propanol; and at the end, 5% DMF–95% 2-propanol. Each fraction was dried in vacuo at 45°C, dissolved in MQW, and examined for IL-6 production-inducing activity. The activity was recovered in fractions with two peaks eluted at hydrophilic and hydrophobic regions. The hydrophobic fractions seemed to contain lipoproteins or lipids.

<table>
<thead>
<tr>
<th>Concentration of ammonium sulfate used to separate proteins (%)</th>
<th>Total amount of protein (mg)</th>
<th>Specific activity ([U/mg of protein])</th>
<th>Total U of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–30</td>
<td>15.7</td>
<td>2.1</td>
<td>33.0 (0.8)</td>
</tr>
<tr>
<td>30–60 (P60)</td>
<td>72.1</td>
<td>58.7</td>
<td>4232.3 (97.0)</td>
</tr>
<tr>
<td>60–100</td>
<td>24.9</td>
<td>3.8</td>
<td>94.7 (2.2)</td>
</tr>
</tbody>
</table>

*One unit of activity is defined as the amount of protein required to induce production of 1 ng of IL-6. Each value is the mean of two separate experiments.

**TABLE 2. Effects of proteinase K and lipoprotein lipase on IL-6 production-inducing activity of P60**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sp act* ([U/mg of protein %])</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>58.7 ± 6.4 (100)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>17.4 ± 2.3 (30)</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>18.0 ± 7.5 (31)</td>
</tr>
</tbody>
</table>

*One unit of activity is defined as the amount of protein required to induce production of 1 ng of IL-6. One-hundred-microliter volumes of fractions obtained from the initial reversed-phase chromatography (60 μg of protein) were pretreated at 37°C for 2 h with 1.2 μg each of proteinase K and lipoprotein lipase, and then 20 μl of the reaction mixture was used for stimulation of HGF. Each value is the mean and standard deviation of three separate experiments.
buffer or 5% 2-propanol. The flow rate was 1.0 ml/min. Each fraction was dried in vacuo at 45°C, dissolved in MQW, and examined for IL-6 production-inducing activity. The activity was recovered in fractions with a molecular mass of 4.1 kDa (Fig. 3). Thus, the active entity in peak C was found to be a 4.1-kDa polypeptide (P4.1).

M. fermentans cells and peak C were spotted onto two nitrocellulose membranes. To avoid nonspecific binding of antibodies, each of the membranes was blocked with 5% skim milk in phosphate-buffered saline. One membrane was then reacted with anti-M. fermentans serum, the other was reacted with preimmune serum. Each of antibodies was detected by horse-radish peroxidase-conjugated goat anti-rabbit immunoglobulin G and a DAB substrate (Vector Laboratories, Inc., Burlingame, Calif.). Anti-M. fermentans serum, but not preimmune serum, reacted with M. fermentans cells and peak C (Fig. 4). The fact that only anti-M. fermentans serum reacted with peak C demonstrated that P4.1 was derived from M. fermentans.

Mycoplasmal lipoproteins activate lymphocytes, monocytes/macrophages, and fibroblasts, and the activity resides in the hydrophilic fractions, peak A (Fig. 2).

The effect of polymyxin B on the IL-6 production-inducing activity of peak A was investigated to eliminate the possibility that the activity was attributable to lipopolysaccharides that might contaminate peak A. HGF were preincubated with polymyxin B (0, 5,000, and 10,000 U/ml) because polymyxin B is an antibiotic that destroys the biological activities of lipopolysaccharides. The activity was not affected by polymyxin B (data not shown).

Properties of the active entities in peak C. Peak C activated macrophages to produce TNF-α and IL-1β (data not shown). Peak C was found to contain substances with amino groups and carbohydrates. The specific activity of peak C calculated on the basis of the amount of amino group-containing substances or carbohydrate-containing substances was about 75-fold higher than that of P60, while the activity calculated on the basis of the amount of carbohydrate-containing substances was lower than that of P60 (Table 3).

The effect of lipoprotein lipase on the IL-6 production-inducing activity of peak C was investigated. A 0.02:1 (wt/wt) reaction mixture of lipoprotein lipase and peak C was treated at 37°C for 2 h and then tested for the activity. It was found that lipoprotein lipase had no effect on the activity of peak C (data not shown).

SDS-PAGE of peak C revealed one dense band with a molecular mass of 4.1 kDa (Fig. 3). Thus, the active entity in peak C was found to be a 4.1-kDa polypeptide (P4.1).

M. fermentans cells and peak C were spotted onto two nitrocellulose membranes. To avoid nonspecific binding of antibodies, each of the membranes was blocked with 5% skim milk in phosphate-buffered saline. One membrane was then reacted with anti-M. fermentans serum, the other was reacted with preimmune serum. Each of antibodies was detected by horse-radish peroxidase-conjugated goat anti-rabbit immunoglobulin G and a DAB substrate (Vector Laboratories, Inc., Burlingame, Calif.). Anti-M. fermentans serum, but not preimmune serum, reacted with M. fermentans cells and peak C (Fig. 4). The fact that only anti-M. fermentans serum reacted with peak C demonstrated that P4.1 was derived from M. fermentans.

Mycoplasmal lipoproteins activate lymphocytes, monocytes/macrophages, and fibroblasts, and the activity resides in the hydrophilic substances in M. fermentans popeptides that had already been characterized (16). However, hydrophilic substances in M. fermentans capable of inducing production of cytokines have not been identified. Therefore, we were very much interested in the active entities in the hydrophilic fractions, peak A (Fig. 2).

Fractionation was carried out by using the following program: at time zero, 0.05% TFA–80% acetonitrile; at 20 min, 0.05% TFA–80% acetonitrile; and at the end, 0.05% TFA–80% acetonitrile. Each fraction was dried in vacuo at 45°C, dissolved in MQW, and examined for IL-6 production-inducing activity (Fig. 2). Abs, absorbance.
N-terminal lipopeptide moieties (16, 22). Lipoproteins are not released in a water-soluble form from intact mycoplasma cells because they are anchored to the cell membranes by the N-terminal lipid moiety. Therefore, we were interested in mycoplasma-derived and hydrophilic substances capable of activating mammalian cells because we thought that hydrophilic substances are capable of interacting with target cells more easily and play more important pathological roles than hydrophobic substances such as lipoproteins. Recently, we have partially purified and characterized hydrophilic substances from *M. salivarium* that are responsible for induction of IL-6 production by HGF (6). In this sense, we were very much interested in the finding that the cultural supernatant of *M. fermentans* induced IL-6 production by HGF. The activity existed in hydrophilic and hydrophobic substances in the cultural supernatant. Hydrophilic substances are possibly lipoproteins or lipopeptides liberated in some way from cell membranes of *M. fermentans* and solubilized in the cultural supernatant, possibly by association with serum albumin in horse serum, an essential ingredient of growth medium. The active entity in hydrophilic substances was found to be a 4.1-kDa peptide, named P4.1, with carbohydrates (Table 3). P4.1 is presumably a product of *M. fermentans* or, less likely, a fragment separated from proteins or lipoproteins in cell membranes of the organism by some enzymes, because the activity increased almost in parallel with viable counts in cultures and reached a maximum at the end of the log phase (Fig. 1).

P4.1 activates TNF-α production by macrophages. MALP-2 has been purified from the cell membrane of *M. fermentans* (16) and is thought to be the N-terminal lipopeptide moiety released from lipoproteins degraded by some proteolytic enzyme. Therefore, it is very likely that the cultural supernatant of *M. fermentans* contains lipopeptides such as MALP-2. Lipopeptides are possibly eluted at the hydrophobic region in reversed-phase HPLC. However, P4.1 was eluted at the hydrophilic region and is resistant to lipoprotein lipase. Therefore, it is speculated that P4.1 is another activator of macrophages.

*M. fermentans* was first isolated (19, 20) from the human lower genital tract and is thought to be a common inhabitant of the genital tract. *M. fermentans* has been suggested to be associated with rheumatoid arthritis (RA) and AIDS because *M. fermentans* was also isolated from RA patients (32), detected in synovial fluid of RA patients by PCR methods (9, 21), and isolated from AIDS patients (10, 14).

Large amounts of cytokines such as TNF-α, IL-1β, IL-6, and IL-8 are produced in the inflamed rheumatoid synovial fluid of RA patients and play crucial roles in the pathophysiology of RA (28). The finding that P4.1 and lipoproteins induce IL-6 production by HGF suggests that they are capable of inducing IL-6 production by synovial fibroblasts.

Many studies on AIDS have suggested that mycoplasmas, including *M. fermentans*, are involved for some reasons as possible cofactors in AIDS pathogenesis. For example, higher titers of antibodies to these mycoplasmas have been detected in human immunodeficiency virus-infected patients than in noninfected individuals (30), and *M. fermentans* has been shown to have the ability to invade host cells (15, 29) and induce TNF-α secretion by monocytes, which is known to activate human immunodeficiency virus replication (18). The finding that P4.1 induces TNF-α production by macrophages suggests that it may play some pathological role in AIDS.

Periodontitis is an inflammatory disorder characterized by bone resorption. IL-6 is an inflammatory cytokine that plays an etiological role in this disease. IL-6 alone does not induce bone resorption by osteoclast formation, but soluble IL-6 receptors trigger the formation in the presence of IL-6 (12). *Porphyromonas gingivalis*, a gram-negative, anaerobic bacterium, is thought to be one of the pathogens in periodontitis. *P. gingivalis* possesses the ability to induce IL-6 production by HGF (27). Baker et al. reported that IL-6 production induced by infection with *P. gingivalis* contributes to alveolar bone loss (1). P4.1 also induces IL-6 production by HGF. In addition, *M. fermentans* has been detected in human saliva (24). This suggests that P4.1 plays some etiological role in periodontitis.

Temporomandibular disorder is a disease in which pain and impaired mandibular movement appear to arise directly from degenerative or inflammatory changes within the temporomandibular joint, but its precise pathogenesis has not been elucidated. It has been suggested that the development of temporomandibular disorder may be traced to a single traumatic event that occurred before the manifestation of symptoms of the disease (26). However, we detected *M. fermentans* in the synovial fluid of patients with temporomandibular disorder by PCR (31). Recently, Henry et al. also reported that they detected some microbes, including *M. fermentans*, in temporomandibular joints (8). Therefore, P4.1 may play an etiological role in temporomandibular disorder. For these reasons, *M. fermentans* may be involved in the pathogenicity of some oral diseases.

This study has demonstrated that *M. fermentans* produces a hydrophilic polypeptide capable of inducing the production of inflammatory cytokines other than lipoproteins.

This work was partially supported by Grants-in-Aid for Scientific Research (C) (10671762), which were provided by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES


Editor: R. N. Moore