Interleukin-8 Secretion of Human Epithelial and Monocytic Cell Lines Induced by Middle Ear Pathogens

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Abstract: Otitis media with effusion (OME) is one of the most common diseases in children. Alloiococcus otitidis, a new Gram-positive bacterial species, was isolated from the middle ear fluid of children with OME; however, the pathogenic role of this bacteria is yet unknown. In this study, the ability of cultured epithelial cell lines (Hep-2 and Hela) and monocytic cell lines (THP-1 and U 937) to secrete chemokine interleukin-8 (IL-8) in response to the A. otitidis organism and three bacterial organisms mainly detected from middle ear fluid in OME, and bacterial cell components was investigated. When stimulated with four viable bacterial cells, epithelial cells and monocytes secreted IL-8 in a time-dependent manner. The monocytes produced significantly higher levels of IL-8 than the epithelial cells. Compared with that by viable bacterial cells, IL-8 secretion by stimulated epithelial cells and monocytes was reduced when the bacteria were heated and treated with glutaraldehyde. With bacterial stimulations, cell treatment of interferon-gamma caused monocytes to increase the induction of IL-8 production, however, the induction of monocye differentiation caused monocytes to reduce the induction of IL-8 production. Furthermore, epithelial cells and monocytes stimulated by four viable bacterial organisms physically separated from cultured cells reduced the induction of IL-8 compared with directly stimulated cells, and monocytes stimulated with soluble extracts prepared from A. otitidis organisms produced IL-8 in a dose-dependent manner. These results suggest that part of the IL-8 stimulation of the A. otitidis organism may exist in a diffusable factor released by the bacteria or soluble components of the bacteria itself.

Key words: Chemokine, Epithelial cell, Monocytic cell, Alloiococcus otitidis

Otitis media with effusion (OME) is frequently observed in childhood, but the etiology has not yet been well clarified. Routine bacterial cultures of middle ear effusion (MEE) have yielded bacterial growth in only 20–30% of OME cases. By using more sophisticated methods such as polymerase chain reaction (PCR), the detection rate could be increased up to 90% (12, 15, 24). The pathogenic bacteria most often encountered include Moraxella catarrhalis, Streptococcus pneumoniae and Haemophilus influenzae.

An unclassified, slow-growing, Gram-positive organism was first isolated from the middle ear fluids of children with persistent OME in 1989 (9). Aguirre and Collins (2) named this newly recognized Gram-positive bacteria Alloiococcus otitidis, and this nomenclature was revised to Alloiococcus otitidis in keeping with the rules of the Bacteriological Code (26). However, in a recent study using PCR for the detection of microbes, A. otitidis was found in over 40% of child OME cases (3, 11) and was more frequently detected than other common pathogens of otitis media (11). There have been reports of the isolation of Alloiococcus otitidis strains which were resistant to both erythromycin and trimethoprim-sulfamethoxazole and relatively resistant to beta-lac-
tams (4). Therefore, this new bacterial species, *A. otitidis*, should be examined to determine whether or not it has a pathogenetic role in the occurrence of otitis media.

It is thought that the primary function of chemokine, interleukin (IL)-8, is to serve as a potent inflammatory mediator attracting and activating polymorphonuclear leukocytes and neutrophils, the latter in particular. It has been reported that IL-8 plays an important role in middle ear inflammation. IL-8 transcripts were frequently detected in both pediatric and adult MEEs (23), and IL-8 was responsible for the recruitment of leukocytes in the tympanic cavity (23). It was reported that the mean level of IL-8 in MEEs from children was higher than that from adults (14). Moreover, the IL-8 concentration in MEEs with bacteria from pediatric otitis media was significantly higher than that in MEEs without bacteria (7, 22). In animal experiments, the mRNA expression of rat IL-8-like-chemokine in rat middle ear mucosa was easily induced after exposure to Gram-positive and -negative bacterial components (19, 28). Therefore, IL-8 induction by middle ear pathogens should be investigated to clarify the pathogenesis of pediatric otitis media.

In this study, the ability of cultured epithelial cell lines and mononuclear cell lines to secrete IL-8 in response to not only three representative pathogens of otitis media but also the *A. otitidis* strain was investigated. We also examined whether indirect contact between cultured cells and bacteria could affect the induction of chemokine secretion and whether cell differentiation and interferon (IFN)-gamma could be involved in upregulating chemokine secretion in mononuclear cells.

**Materials and Methods**

**Bacterial strains and preparation.** The bacterial strains used in this study were *Alloioococcus otitidis, Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*. *A. otitidis* strain was NCFB1118 and the other three bacterial strains were clinical isolates. *A. otitidis, S. pneumoniae* and *M. catarrhalis* were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich., U.S.A.) and *H. influenzae* was grown in chocolate II agar (Nippon Becton Dickinson, Co., Tokyo) at 37 C for 2 days in 5% CO\(_2\)/95% air. Cultures were centrifuged and washed three times in antibiotic-free phosphate-buffered saline (PBS). The killed bacteria were produced by fixation with 0.25% (vol/vol) glutaraldehyde (GA) (Nacalai Chemicals, Ltd., Kyoto, Japan) at room temperature for 1 hr, or heat-killed at 56 C for 30 min. Killed bacteria were washed three times in PBS with antibiotics. Aliquots of the viable or killed bacterial suspensions were adjusted to 3.0×10\(^6\) organisms/ml. The numbers of bacteria were determined using the McFarland method.

**Cell culture and stimulation.** Human epithelial cell lines, HEp-2 (ATCC CCL 23) and Hela (ATCC CCL 2), and human mononuclear cell lines, THP-1 (ATCC CRL 1593) and U 937 (ATCC TIB 202), were obtained from the American Type Culture Collection, Rockville, Md., U.S.A. Cells were grown by suspension in 5% CO\(_2\)/95% air in RPMI 1640 medium (Life Tech., Grand Island, N.Y., U.S.A.) with 0.1% (vol/vol) 2-mercaptoethanol (Life Tech.,), supplemented with 10% (vol/vol) fetal calf serum (FCS) (ICN Biomedicals, Inc., Aurora, Ohio, U.S.A.) containing 100 U/ml penicillin and 100 µg/ml streptomycin.

HEp-2 cells and Hela cells were seeded in 24-well tissue culture plates (Becton Dickinson, Co., Lincoln Park, N.J., U.S.A.) at 2×10\(^5\) cells per well at a volume of 1 ml per well and cultured for 2 days. Prior to stimulation, each well was washed three times with 1 ml of fresh tissue culture medium with or without antibiotics as indicated. THP-1 and U 937 cells (1.0×10\(^6\)/ml) were not-treated or pretreated for 24 hr with 1.2% DMSO and incubated in the presence or absence of recombinant human IFN-gamma (100 ng/ml) for 16 hr. Mononuclear cells were distributed to round-bottomed tissue culture tubes (Becton Dickinson, Co.) at 1.0×10\(^6\) cells per tube in a volume of 1 ml medium.

Bacterial cells were added to the cultured cells at a bacterium/cell ratio of 100:1 in a 1-ml volume. Antibiotic-free medium was used in the experiment for viable bacteria. After incubation at 37 C in 5% CO\(_2\)/95% air, supernatants were collected at various time intervals.

**Blood mononuclear cells.** Human peripheral blood mononuclear cells (PBMC) were isolated from heparin-anticoagulated peripheral blood from healthy donors by Ficoll-Hypaque gradient centrifugation. PBMC were washed twice and resuspended in RPMI 1640 medium with 0.1% 2-mercaptoethanol supplemented with 10% FCS and containing 100 U/ml penicillin and 100 µg/ml streptomycin. For cytokine production, PBMC (1.0×10\(^6\)/ml) were incubated in the presence of recombinant human IFN-gamma (100 ng/ml) for 16 hr. Cells were distributed to round-bottomed tissue culture tubes at 1.0×10\(^6\) cells per tube in a volume of 1 ml medium, and then bacteria was added to the cultured cells at a bacterium/cell ratio of 1:1 in a 1-ml volume.

**A two-chamber system.** We used a two-chamber system in the same experiment to test whether *Alloioococcus otitidis* and the other three bacterial organisms have an effect per se, or through the release of some materials, on IL-8 production in the epithelial cell line and two mononuclear cell lines. HEp-2 cells were incubated as previously described. DMSO-treated IFN-gamma plus THP-1 cells and U 937 cells were also seeded in 24-
well tissue culture plates at $2 \times 10^5$ cells per well in a volume of 1 ml per well and cultured for 2 days. Prior to stimulation, each well was washed three times with 1 ml of fresh tissue culture medium without antibiotics, and then bacterial cells were added to the cultured cells at a bacterium/cell ratio of 100:1 in a 1-ml volume in the presence or absence of cell culture inserts (Becton Dickinson, Co., Franklin Lakes, N.J., U.S.A.). Supernatants were collected at 16 hr after stimulation.

**Soluble extract of bacterium.** Bacterial cells of Alloiococcus otitidis (NCIB 118) were grown in Todd-Hewitt broth for 3 days, and then antibiotics (1,000 U/ml of penicillin G and 1,000 µg/ml of streptomycin) were added to the culture. After treatment of the cells with antibiotics for 90 min, the bacterial cells were harvested by centrifugation (3,000 rpm for 30 min) and washed three times with PBS. The resuspended cells in PBS were disrupted with a Branson cell homogenizer (Branson Model 250 Sonifer, Branson, Conn., U.S.A.), and the unbroken cells were removed by centrifugation (12,000 rpm for 10 min). Supernatants were collected as soluble extract of bacterium. Protein concentration was measured by Bio-rad protein assay (Bio-Rad Lab., Richmond, Calif., U.S.A.).

**Quantification of IL-8.** Various cell lines were incubated in the presence and absence of various bacterial cells, and then the supernatants were collected at various time intervals as noted above. The IL-8 in the resulting supernatants was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn., U.S.A.) following the manufacturer’s protocol.

**Results**

**Induction of Secretion of IL-8 by Alloiococcus otitidis Strains and Other Bacterial Strains**

In this study, a ratio of 100 bacterial cells per cultured cell was routinely employed based on the results of a preliminary study (data not shown). When stimulated with four viable bacterial cells, epithelial cell line HEp-2 and monocytes cell line THP-1 secreted IL-8 in a time-dependent manner (Fig. 1, A and B). Of the four cell lines tested, the monocyte cell line THP-1 and U 937 produced significantly higher levels of IL-8 than the epithelial cell lines HEp-2 and Hela (Fig. 2). Furthermore, in the monocyte cell lines, THP-1 cells produced higher levels of IL-8 than U 937 cells (Fig. 2B), and in the epithelial cell lines, IL-8 levels produced by HEp-2 cells were higher than those by Hela cells (Fig. 2A). On the other hand, PBMC produced higher levels of IL-8 than the two epithelial cell lines and U 937 cells in spite of a bacterium/cell ratio of 1:1 (Fig. 2B).

Compared with the result using viable bacterial cells, IL-8 secretion by stimulated HEp-2 cells was reduced when the bacteria were killed by heating at 56 C for 30 min. Bacterial cells treated with 0.25% glutaraldehyde showed no induction of IL-8. This inhibitory effect of IL-8 production by the treatment of bacterial cells in the HEp-2 cells did not show a significant difference among the four bacteria (Table 1).

Meanwhile, THP-1 cells produced high levels of IL-8 protein synthesis when stimulated with viable bacterial cells (Table 2A). Compared with that using viable bacterial cells, IL-8 secretion by stimulated THP-1 cells was reduced without H. influenzae when the bacteria were pretreated with heat-killing or GA fixation. The level of IL-8 production was low using S. pneumoniae (Table 2A). Furthermore, THP-1 cells pretreated with IFN-gamma tended to produce higher levels of IL-8 protein than non-treated THP-1 cells, and IL-8 secretion in DMSO-treated THP-1 cells was reduced compared with that in DMSO-non-treated cells (Table 2B).
To examine whether IL-8 expression was triggered by direct contact between cultured cells and viable bacteria or a diffusable factor released by the bacteria, we performed experiments in a two-chamber system, in which *A. otitidis* and the other bacterial organisms were physically separated from the epithelial cells and monocytic cells by cell culture inserts. IL-8 production by stimulated HEp-2 cells was reduced to a greater extent when live bacteria were physically separated from cells when compared with IL-8 secretion produced by direct contact between cells and viable bacteria (Table 1). On the other hand, similar results were obtained with the monocytic cell lines THP-1 and U 937 (Table 3), but there was a difference between the two cell lines; namely, THP-1 cells stimulated with physically separated Gram-positive bacteria showed less induction of IL-8 secretion, while U 937 cells stimulated with physically separated Gram-negative bacteria showed less induction of IL-8 secretion (Table 3). When stimulated with a soluble extract of *A. otitidis*, monocytic cell line THP-1 (pretreated both with DMSO and with IFN-gamma) secreted IL-8 in a dose-dependent manner after stimulation (Fig. 3).

**Discussion**

It has been reported that IL-8 plays an important role in middle ear inflammation. IL-8 and other proinflammatory cytokines were detected in MEE from patients with OME, and this suggested that the pathogenesis of OME in children may differ from that in adults (13, 14, 21). These cytokines were intimately involved in the inflammatory cascade in the middle ear. IL-8 was produced during acute infection of the middle ear, and these polymorphonuclear leukocyte-related inflammatory substances may play an important role in delaying recovery or in the recurrence of acute otitis media (7). Furthermore, the expression of IL-8 gene in MEEs of children and adults with OME has been reported (23). The IL-8 concentration in MEE with bacteria was significantly higher than in MEE without bacteria (22). Moreover, the expression of an IL-8-like chemokine gene was easily induced by bacterial components in rat middle ear mucosa *in vivo* (19, 28).

Various types of epithelial cells can participate in local cytokine networks and regulate inflammatory events by synthesizing and secreting various cytokines and chemokines that communicate with infiltrating inflammatory cells and structural cells. Interaction between cytokine products derived from middle ear mucosa and effector cells may directly contribute to the pathogenesis of inflammation.

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**Table 1. Effect of treatments of various bacterial cells on induction of IL-8 in cultured Hep-2 cells**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>IL-8 (pg/ml)</th>
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<tr>
<td></td>
<td>Viable</td>
</tr>
<tr>
<td><em>A. otitidis</em></td>
<td>477.8± 79.5</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>766.4±319.5</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>625.8±18.2</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>534.0±6.9</td>
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</tbody>
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Values represent the means of two determinations ± standard deviation.

The value using various treated bacteria/viable bacteria.
of acute otitis media and otitis media with effusion. The normal middle ear mucosa is constructed by a thin epithelial layer with less submucosal components as compared to the mucosae of other respiratory tracts. This anatomical construction of the middle ear mucosa may contribute to modify the inflammatory response in the middle ear. Therefore, the production of inflammatory cytokines, induced by middle ear pathogens, should be studied regarding not only monocytes/macrophages but also epithelial cells.

In the present study, we assessed whether the A. otitidis strain and three representative pathogens detected in OME showed different patterns in the induction of IL-8 secretion by epithelial cells and monocytes. The A. otitidis strain and three bacterial strains induced IL-8 production in a time-dependent manner in both Hep-2 cells and THP-1 cells. Despite the differences in the amounts of IL-8 produced, the bacterial kinetic responses in IL-8 induction were similar regardless of cell line origin. It has been reported that H. influenzae strains induced IL-6 and IL-8 production in human epithelial cell line in vitro (5, 25). It has also been reported that H. influenzae strains could induce IL-8 production in leukocytes and monocytes (5, 25).

Meanwhile, following exposure to Helicobacter pylori cells, epithelial cell lines secreted IL-8 with a difference between cells of gastric and nongastric origin (16).
These strains could induce not only IL-8 expression but also the expression of other cytokines such as MCP-1, IL-6 and IL-10 in both epithelial cells and monocytes. *E. coli* activated response of cytokines such as IL-1 alpha, IL-6 and IL-8 in the epithelial cell lines (1). Other varieties of bacteria, such as *Salmonella dublin*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Shigella dysenteriae* also induced IL-8 secretion by epithelial cells (8, 18), and *Mycobacterium tuberculosis* was a more potent inducer of IL-8, but not of TNF, than LPS *in vitro* in monocytic cell lines (10). However, the stimulating ability of IL-8 production by *A. otitidis* in epithelial cells and monocytes remains unknown. In this study, it was found that the stimulation of *A. otitidis* strain induced IL-8 expression and production in the epithelial cell line, HEp-2, monocytic cell lines, THP-1 and U 937, and PBMC to a similar degree as other representative middle ear pathogens.

In *Helicobacter pylori* cells, IL-8 secretion in stimulated epithelial cells was reduced when the bacteria were pretreated with formaldehyde and heating (16). Similarly, viable bacteria had a much stronger stimulating ability than bacterial cells killed by heat- or glutaraldehyde-treatment in both epithelial cells and monocytes, suggesting that either the IL-8-stimulating factor was labile or that it was essential for active bacterial metabolism. It has been reported that IFN-gamma in combination with TNF-alpha caused a dose-dependent induction of beta chemokine RANTES and MCP-1 in colonic epithelial cell lines, while IFN-gamma had no significant effect on the alpha chemokine IL-8 secretion stimulated by TNF-alpha (27). Leng and Elias (20) showed that IFN-gamma had a significant effect on SAC stimulated IL-12 secretion in THP-1 cells. In this study, THP-1 cells pretreated with IFN-gamma tended to produce higher levels of IL-8 protein than non-treated THP-1 cells. Indoh et al (17) reported that cell differentiation of U 937 cells by TPA (12-O-tetradecanoyl-phorbol-13-acetate) had a significant effect on the induction of IFN-beta production, while cell differentiation of THP-1 cells had no effect on it.

It has been reported that when physically separated from macrophages, viable *Brucella* impaired TNF-alpha production in *E. coli*-infected U 937 cells (6). In this study, both epithelial cells and monocytes physically separated from viable bacterial organisms produced lower levels of IL-8 than non-separated cells. However, there was a difference between two monocytic cell lines; THP-1 cells stimulated with physically separated Gram-positive bacteria showed less induction of IL-8 secretion, while U 937 cells stimulated with physically separated Gram-negative bacteria showed less induction of IL-8 secretion, suggesting that a diffusible factor released by the bacteria could have induced the IL-8 production and this factor could be different between Gram-positive and -negative bacterial organisms. Furthermore, THP-1 cells stimulated with soluble extracts prepared from *A. otitidis* strains produced IL-8 in a dose-dependent manner, and cell differentiation of THP-1 cells by DMSO had no effect on IL-8 production. These results suggest that part of the IL-8 stimulation of the *A. otitidis* organism may exist in a diffusible factor released by the bacteria or soluble components of the bacteria itself.

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References


