Effect of *Mycoplasma pneumoniae* Lysate on Interleukin-8 Gene Expression in Human Respiratory Epithelial Cells*

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**Study objectives:** *Mycoplasma pneumoniae* is a common cause of lower respiratory disease. Several studies have suggested that respiratory infection by *M pneumoniae* is associated with reactive airway disease and asthma. Interleukin (IL)-8 has been suggested to have a role in the pathogenesis of the allergic inflammation of bronchial asthma, and is well known to be expressed in bronchial epithelial cells.

**Measurements:** An examination was carried out into the effect of *M pneumoniae* lysate (MPL) and the role of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinase (ERK) on IL-8 expression in human lung epithelial cells. A549 cells were seeded at a density of 5 × 10^4^ cells per well and incubated in basal medium for a further 24 h. IL-8 levels were determined by an enzyme-linked immunosorbent assay. MAPK phosphorylation was assessed by Western blotting.

**Results:** In A549 cells, MPL induced IL-8 release in a time- and dose-dependent manner. Pretreatment with PD 98059, which blocks the activation of MAPK/ERK kinase 1, inhibited MPL-induced IL-8 production by 64.4% at 25 μmol/L. Stimulation of A549 cells by MPL also caused an increase in the activity of ERK, compared with the nonstimulated cells. The MPL stimulation had no effect on the activities of p38.

**Conclusion:** These observations suggest that activation of ERK by MPL may be one of the mechanisms that result in an increase of the production of IL-8.


**Key words:** epithelial cells; interleukin-8; mitogen-activated protein kinases; *Mycoplasma pneumoniae*

**Abbreviations:** ERK = extracellular signal-regulated kinase; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; IL = interleukin; JNK = c-Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MEK = mitogen-activated protein/extracellular signal-regulated kinase; MPL = *Mycoplasma pneumoniae* lysate; NF = nuclear factor; PCR = polymerase chain reaction; RT = reverse transcriptase; TBS-T = Tris-buffered saline solution plus Tween 20

*Mycoplasma pneumoniae* is a common cause of lower respiratory disease, especially in children and young adults. Approximately 10% of patients with Mycoplasma infection will acquire major respiratory disease, the features of which are not distinct enough to allow an accurate diagnosis without recourse to serologic disease investigation.1 Several studies2,3 have suggested that respiratory infection by *M pneumoniae* is associated with reactive airway disease and asthma. *M pneumoniae* has been isolated from the respiratory tract of up to 20 to 25% of asthmatics experiencing acute exacerbations.4,5 It was present in the lower airways of chronic, stable asthmatics, with greater frequency than in control subjects.6,7 There are also studies8,9 that show *M pneumoniae* could induce bronchial hyperresponsiveness that may be transient or persistent. Additionally, a previously healthy patient with Mycoplasma pneumonia subsequently had an initial onset of bronchial asthma that was attributed to the pneumonia.10 More recently, it was established that murine models of *M pneumoniae* respiratory infection can demonstrate a link to reactive airway disease and asthma.11,12

Interleukin (IL)-8 is a neutrophil chemotactic and activating peptide13 and has been suggested to have a role in the pathogenesis of allergic inflammation of...
bronchial asthma,\textsuperscript{14,15} and is well known to be expressed in bronchial epithelial cells.\textsuperscript{16} \textit{M pneumoniae} typically infects ciliated epithelial cells in the respiratory tract, and colonization is mediated by the attachment tip structure of \textit{M pneumoniae} cells.\textsuperscript{17} Several reports\textsuperscript{18,19} suggested that \textit{M pneumoniae} infection induces proinflammatory cytokine expression, such as IL-8 in human nasal and lung epithelial cells. Furthermore, a recent study\textsuperscript{20} proposed that \textit{M pneumoniae} infection may contribute to the pathogenesis of chronic asthma by inducing RANTES (regulated on activation, normal T-cell expressed and secreted) and tumor growth factor-\(\beta_1\) in airway epithelial cells. However, the precise mechanisms regulating the expression of the gene encoding these chemokines are poorly understood. In the current study, the effect of \textit{M pneumoniae} lysate (MPL) and the role of mitogen-activated protein kinases (MAPKs), known to modulate transcription factor activities on IL-8 expression in human lung epithelial cells, were examined.

METHODS AND MATERIALS

Preparation of MPL

To prepare consistent \textit{M pneumoniae} stock for use in experiments, strain 15531 (American Type Culture Collection; Rockville, MD) was grown in Chanock modified medium.\textsuperscript{21} \textit{M pneumoniae} cells in exponential growth phase were aliquoted and centrifuged at 20,000 \(g\) for 30 min. The pellet was washed and resuspended with phosphate-buffered saline solution. A cell extract was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin. The cell lysate was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin. The cell lysate was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin. The cell lysate was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin. The cell lysate was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin.

Preparation of Cell Lysates and Western Blot Analysis

A549 cells were grown in an appropriate serum-free, basal medium for 24 h, as described previously, and exposed to varying concentrations of MPL at different times during culture. Culture supernatants were collected, centrifuged at 12,000 \(g\) for 5 min at 4°C, and then stored frozen. The IL-8 concentration was determined with the Quantikine Human IL-8 Immunoassay (R&D Systems; Minneapolis, MN) according to the instruction of the manufacturer. The sensitivity of the assay was <10 pg/mL.

Total RNA was isolated from A549 cells using RNeasy Mini Kit (Qiagen; Valencia, CA). The isolated RNA was dissolved in distilled water and quantified. To prepare complementary DNA, 3 \(\mu\)g of total RNA was reverse transcribed using 1 \(\mu\)L of 100 \(\mu\)M dNTPs, 200 U of Superscript II reverse transcriptase (RT) (GibcoBRL; Gaithersburg, MD). The reaction was incubated at 42°C for 50 min, and terminated by elevating the temperature to 80°C for 10 min. For polymerase chain reaction (PCR), 2 \(\mu\)L of the RT product was used in a total volume of 50 \(\mu\)L containing the following: 1 U of Taq polymerase (TaKaRa; Shiga, Japan), forward and reverse primers (10 pmol each). The following sequence was performed on a thermocycler for each PCR reaction: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension phase at 72°C for 7 min. PCR for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), used as the internal control, was performed on each sample. The sequences of primers used in this experiments were as follows: IL-8 (forward): 5'-AGA TAT TGC ACC GGA GAA-3'; IL-8 (reverse): 5'-GAA ATA AAG GAG AAA CCA-3'; GAPDH (forward): 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH (reverse): 5'-TCC ACC CTG TTG CTG TA-3'. The PCR products were separated on a 1% agarose gels with ethidium bromide, visualized by ultraviolet illumination, and photographed.

Stimulation of Epithelial Cells and Measurement of IL-8 Protein

A549 cells were grown in an appropriate serum-free, basal medium for 24 h, as described previously, and exposed to varying
horseradish peroxidase diluted 1:2,000 in TBS-T was applied to the membrane for 60 min. The membrane was washed again three times with TBS-T and visualized by an enhanced chemiluminescence system (Cell Signaling Technology).

**P1 Adhesin Protein Blockage Assay**

A mouse anti-M pneumoniae monoclonal antibody (Chemicon International; Temecula, CA), which targets the P1 adhesin protein, was incubated with 5 μg/mL of MPL at a 1:10, 1:100, or 1:1,000 ratios for 2 h at room temperature. A549 cells were then stimulated with the mixture, and IL-8 release was assayed after 24 h.

**Statistical Analysis**

Data were expressed as mean ± SEM from three to five independent experiments. Statistical significance between treatment and control groups was assessed by Student t test; p < 0.05 was considered significant.

**RESULTS**

**MPL Induces IL-8 Release From A549 Cells**

The first examination conducted was the production of IL-8 by A549 cells following stimulation with MPL at 2.5 μg/mL, 5 μg/mL, and 10 μg/mL. IL-8 secretion was clearly detected in a dose- and time-dependent manner over a 24-h period, when compared with medium controls. Maximal production was reached with 10 μg/mL of MPL (Fig 1). After 1, 2, 6, 10, and 24 h of coculture with 10 μg/mL of MPL, IL-8 production was increased by 3.75 ± 0.34 pg/mL, 24.22 ± 3.03 pg/mL, 152.24 ± 7.30 pg/mL, 242.52 ± 18.90 pg/mL, and 623.40 ± 10.77 pg/mL, respectively. IL-8 release increased with time. Maximum production was observed at 24 h on each concentration, but had not reached a plateau by this time. Additionally, when tested by RT-PCR with GAPDH as an internal control, it was found that MPL stimulation of A549 cells resulted in elevation of IL-8 messenger RNA, with the signal becoming gradually stronger until 24 h (data not shown).

Intracellular MAPK Pathways Are Involved in IL-8 Release Induced by MPL

To examine the functional relevance of one or more intracellular signaling pathways on the activation of respiratory epithelial cells in response to MPL, an investigation was conducted to determine whether IL-8 production by A549 cells, induced by MPL, is mediated through activation of MAPKs, which are known to participate in multiple cellular functions. As shown in Figure 2, preincubation of A549 cells with PD98059, a specific inhibitor of MEK significantly, but not completely, inhibited MPL-induced IL-8 production by 64.4% (p < 0.05). However, the p38 MAPK inhibitor SB202190 did not inhibit significantly IL-8 release in response to MPL.

To prove the intracellular activation of MAPK signaling pathways, an examination of the phosphorylation of ERK or p38 MAPK in MPL-stimulated A549 cells was conducted. By Western blot analysis, both phosphorylated forms of ERK1 and ERK2 were maximally detected in A549 cells at 30 min after incubation of MPL. In contrast, phosphorylated p38 was not observed in MPL-stimulated A549 cells (data not shown).

**Figure 1.** The effects of MPL on IL-8 production by human lung epithelial cells. A549 cells were stimulated with different concentrations of MPL (2.5 to 10 μg/mL) or medium alone at 37°C. After each incubation, culture supernatants were collected and assayed for IL-8 by enzyme-linked immunosorbent assay. The data represent the mean ± SEM from four separate experiments.

**Figure 2.** The effects of MAPK inhibitors on MPL-induced IL-8 release from human lung epithelial cells. A549 cells were preincubated with PD98059 (25 μM) or SB202190 (25 μM) for 30 min at room temperature before the addition of 5 μg/mL MPL antigen. After 24 h of culture, supernatants were collected and evaluated for IL-8 presence. The data represent the mean ± SEM from four separate experiments. *p < 0.05 vs MPL alone.
The P1 Adhesin Protein of MPL Is Not Important for IL-8 Release

To examine the importance of cytadherence for proinflammatory cytokine production, an anti-P1 monoclonal antibody that can inhibit the attachment of Mycoplasma pneumoniae to respiratory epithelium was used. When A549 cells were stimulated with MPL preincubated with anti-P1 antibodies at a dilution of 1:10, 1:100, or 1:1,000, IL-8 was not significantly inhibited (data not shown).

Discussion

The signaling mechanism leading to IL-8 production from M pneumoniae infection has not been clearly defined. In the present report, it was determined that stimulation of human respiratory epithelial cells by MPL altered the activities of various MAPKs. Incubation of A549 cells with MPL resulted in significant production of IL-8 from A549 cells. Additionally, it was found that the activity of ERK was increased, whereas the activity of p38 was not changed. Furthermore, pretreatment of A549 cells with MAPK inhibitors prior to incubation with MPL was tested whether the production of IL-8 could be inhibited. At 25 μM, PD98059 inhibited the increased of IL-8 by 64%, whereas SB202190 had little effect on IL-8 production. The IL-8 gene is regulated transcriptionally, post-transcriptionally, and translationally. Virtually all stressful and proinflammatory stimuli known to induce IL-8 production activate a number of protein kinases, which in principal have the capacity to modulate nuclear factor (NF)-κB or activator protein-1 activity. Activator protein-1 is activated by MAPKs. Three MAPKs pathways contribute to IL-8 gene expression: the ERK, the c-Jun N-terminal kinase (JNK), and the p38 MAPK cascades.

Although the role of NF-κB, JNK, and p38 pathways in IL-8 gene regulation has been analyzed in detail, information about the role of the MKK1 signaling molecule including the ERK pathway is very limited. Based on use of the MEK-1 inhibitors PD98059 and U0126, there is some evidence that the ERK pathway contributes to IL-8 expression. Holtmann et al. found that epidermal growth factor, a physiologic activator of ERK, weakly induces IL-8 in a JNK- and NF-κB-independent manner. Additionally, they showed that expression of a constitutively active mutant of MEK1 caused some IL-8 transcription but failed to induce significant IL-8 protein. They proposed that this data suggested that the ERK pathway, on its own, is not a very potent inducer of IL-8 but has the potential to contribute to IL-8 induction stimulated by NF-κB and other pathways. Although Chmura et al. reported that mycoplasma membrane fraction activated all three isoforms of the MAPKs, the results of the present study suggests that the activation of ERK may be one of the mechanisms that increased IL-8 production in A549 cells during M pneumoniae infection, and that effect may be at the protein regulation steps of IL-8. Similarly, during infection of lung epithelial cells by respiratory syncytial virus, the increased activity of ERK was found, whereas the activities of p38 and JNK were not changed.

Cytadherence of M pneumoniae to the respiratory epithelium is regarded as an essential primary step in tissue colonization and subsequent disease pathogenesis. Yang et al. reported that IL-1β induction was strongly inhibited when A549 cells were infected with M pneumoniae, preincubated with anti-P1 antibodies. Incubation of A549 cells with anti-P1 antibodies before MPL treatment was carried out in the present study, but IL-8 production was not inhibited. Although MPL was used instead of live M pneumoniae, these results suggest that some other mechanisms may be involved in the production of proinflammatory cytokines.

Conclusion

It has been demonstrated that MPL induces activation of human respiratory epithelial cells directly and that ERK plays an important role in signal transduction in M pneumoniae-induced IL-8 production. These findings indicate that M pneumoniae at the inflammation site may play an important role in recruiting neutrophils into an acutely inflamed site. Further study for the characterization and identification of the putative effector molecules that transmit M pneumoniae-induced ERK activation will be needed.

References

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