Release of Cytokines by Human Nasal Epithelial Cells and Peripheral Blood Mononuclear Cells Infected with *Mycoplasma pneumoniae*

MIKHAIL Y. KAZACHKOV,*[,1] P.C. HU,† JOHNNY L. CARSON,† PAULA C. MURPHY,† FREDERICK W. HENDERSON,† AND TERRY L. NOAH†

Department of Pediatrics, Maimonides Medical Center, Brooklyn, New York; and †Department of Pediatrics, The University of North Carolina, Chapel Hill, North Carolina 27599-7220

***Mycoplasma pneumoniae* (*Mp*) infection is associated with asthma exacerbation in children. We hypothesized that Mp infection may cause airway inflammation by inducing the release of cytokines by respiratory epithelial cells. The levels of chemokines interleukin-8 (IL-8) and released upon activation, normal t cell expressed and secreted (RANTES) released by nasal epithelial cell (NEC) cultures established from asthmatic and nonasthmatic children were measured by ELISA at 4, 24, 48, and 72 hr after cells were inoculated with *Mp*, and were compared with baseline release of these factors. The presence of MP on apical membranes of NEC after infection was confirmed by transmission electron microscopy, and adherence was shown to be inhibited by erythromycin. *Mp* infection did not alter NEC release of IL-8 or RANTES at any time point. In contrast, tumor necrosis factor α (TNF-α) stimulated increased IL-8 at all time points, and respiratory syncytial virus (RSV) infection stimulated RANTES release at 48 and 72 hr by NEC. These results were not significantly different between NEC from asthmatic and nonasthmatic children. As a comparison, peripheral blood mononuclear cells from normal human volunteers were also incubated with *Mp* and had significantly increased release of IL-2, IL-6, and TNF-α. We conclude that *Mp*, unlike viral pathogens such as RSV, is unlikely to directly stimulate early airway surface cytokine responses via mechanisms involving epithelial cells. We speculate that the chronic presence of mononuclear cells at the airway surface of asthmatics provides a target for *Mp*-triggered cytokine production.** [Exp Biol Med Vol. 227(5):330–335, 2002]

Key words: *Mycoplasma pneumoniae;* cytokines; epithelium; mononuclear; asthma

This study was supported by the National Institutes of Health (grant no. P50 HL56395) and by the University of North Carolina Center for Environmental Medicine and Lung Biology (Philip A. Bromberg, MD, Director; U.S. Environmental Protection Agency Cooperative Agreement CR 817643).

[1] To whom requests for reprints should be addressed at Maimonides Medical Center, 4802 10th Avenue, Brooklyn, NY 11219. E-mail: Mkazak@aol.com

Received November 21, 2001.
Accepted January 4, 2002.

1535-3702/02/2275-0330$15.00

Mycoplasma pneumoniae (*Mp*) infection occurs frequently in children and produces a variety of acute respiratory illnesses (1, 2). Several studies suggest that *Mp* may be a trigger for asthma exacerbation, or may perhaps play a role in the maintenance of the asthma state (3, 4). However, the mechanisms responsible for these effects are not clear.

Viral agents such as respiratory syncytial virus (RSV), parainfluenza virus, and rhinovirus promote the release of cytokines by respiratory epithelial cells *in vitro*. We and others have previously shown that RSV infection of human airway epithelial cells causes increased production of cytokines including the chemokines interleukin-8 (IL-8) and released upon activation, normal t cell expressed and secreted (RANTES) (5, 6). These factors are major chemotactic factors for neutrophils (IL-8) and monocytes, lymphocytes, and eosinophils (RANTES). The latter cell types are particularly associated with airway inflammation in asthmatics. The viral pathogens shown to induce these chemokines are also common triggers of clinically significant asthma exacerbations (7–9). Thus, it is possible that direct induction of epithelial chemokines is a primary event triggering increased airway reactivity in the setting of acute respiratory viral infection.

Like the common viruses associated with asthma exacerbation, *Mp* exhibits tropism for airway epithelium. *Mycoplasma hominis* has previously been reported to induce cytokine production by respiratory epithelial cells (10), and *Mp* and other Mycoplasma species have been shown to induce release of several cytokines by human peripheral blood mononuclear cells (PBMC) (18). However, there are no published reports investigating *Mp*'s effects on human respiratory epithelial cytokine production. We hypothesized that *Mp* induces cytokine production by human epithelial cells in a manner similar to RSV. Thus, the goal of this study was to measure the production of IL-8 and RANTES by human epithelial cells infected by *Mp in vitro*.

Materials and Methods

Growth of *Mp*. Monolayer cultures of virulent *Mp* (M129-B10) were grown on glass in Hayflick medium as previously described (22). After incubation at 37°C in 5% CO_2 for 48 hr, Mp were gently scraped into 2.5 to 5.0 ml of either fresh Hayflick medium supplemented with 20% horse serum and 10% yeast dialysate, or modified Eagle's medium (MEM) supplemented with 20% fetal calf serum (FCS) and 10% yeast dialysate. Clumps of Mp were dispersed by passing the suspension through a 25-gauge needle three times. Mp organisms for PBMC stimulation were grown in a similar manner. However, prior to harvest, glass-attached Mps were rinsed with RPMI 1640 to remove Hayflick medium. Mp cells were then scraped from the glass surface into RPMI medium with 10% human albumin and were frozen at −70°C. Viability of the organisms after one freeze-thaw cycle was verified by growth in Hayflick medium.

***Mp* Infection of Nasal Epithelial Cells (NEC).** NEC were obtained by brushing the inferior nasal turbinate of children with asthma and nonasthmatic sibling controls enrolled in a study approved by the University of North Carolina Committee of the Protection of the Rights of Human Subjects. The diagnosis of asthma was established using clinical criteria (history of wheezing episodes and physician diagnosis of asthma within the past 2 years). All asthmatic subjects (six of six) and three of five nonasthmatic subjects were atopic. All atopics had histories of seasonal rhinitis, but none had active rhinitis at the time NEC were brushed. Subjects were not using nasal or systemic corticosteroids at the time the brushings were obtained.

The technique for NEC culture has been previously described (11). In brief, brushed cells were dispersed in 0.5 mg/ml dithiothreitol (Sigma, St. Louis, MO) in PBS and were maintained in plastic culture flasks in serum-free growth media with penicillin-G (BEGM; Clonetics, San Diego, CA). Cultures were grown to near confluence and were then detached from the plastic by incubation with 0.05% trypsin and 0.02% EDTA (JRH Biosciences, Lenexa, KS), and seeded onto vitrogen-coated filters at a density of approximately 5×10^5 cells/24-mm diameter support (Transwell-Col; Costart, Cambridge, MA). Cultures on Transwells were incubated in basolateral media consisting of 1:1 DMEM-H (Invitrogen, Carlsbad, CA) and BEGM. After incubation on Transwells for 3 to 4 days, the cultures were confluent and were used in experiments.

A 0.25-ml *Mp* inoculum containing approximately 2.5×10^{10} plaque forming units (pfu) of *Mp* (multiplicity of infection approximately 1×10^4) was added to the apical surface of NEC on Transwells and was incubated for 2 hr. The NEC cultures were washed two times with cell culture media to remove unattached organisms, and then fresh cell culture media were placed in Transwells. Supernatants were collected at 4, 24, 48, and 72 hr after inoculation of NEC and were frozen at −80°C until cytokine assays were performed. Fresh cell culture media were added to all NEC after each time point so that data represent cytokines accumulated in supernatants only since the previous time point. NEC stimulated with TNF-α (20 ng/ml) or RSV (multiplicity of infection 0.8) were used as positive controls. Negative controls consisted of NEC incubated with media alone, or cultures inoculated with *Mp* in the presence of erythromycin (80 μg/ml).

Isolation of Human PBMC. To enable comparison with previously published reports (18), PBMC were also incubated with *Mp*, and cytokine responses were assessed. Heparinized blood was obtained from healthy young adult volunteers for isolation of PBMC. Plasma was separated from blood by centrifugation, then PBMC were isolated by gradient centrifugation using Histopaque-1077 (Sigma). PBMC were brought to a concentration of 1×10^6/ml in serum-free RPMI 1640. *Mp* was added to PBMC at the same multiplicity of infection as in the NEC experiments above, incubated for 2 hr, then autologous plasma was added to cultures at a final concentration of 10%. Supernatants were collected at 24 and 48 hr after *Mp* incubation for cytokine assays. Positive controls consisted of PBMC stimulated with 0.5 μ/ml *Escherichia coli* lipopolysaccharide (LPS).

Cytokine Assays. Cytokines in cell culture supernatants were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN) and were performed according to the manufacturer's instructions. For NEC experiments, IL-8 and RANTES were measured as per our previous studies with RSV (5, 11). For PBMC experiments, IL-2, IL-6, and TNF-α were measured as per previous reports (18). The correlation coefficient for standard curves was >0.98 for all assays. Samples with cytokine concentrations outside assay dynamic ranges were diluted and re-assayed.

Electron Microscopy. For transmission electron microscopy (TEM) of NEC cultures to confirm attachment of *Mp*, NEC on Transwells were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2). Samples were rinsed three times in 0.1 *M* phosphate buffer, and were then post-fixed for 1 hr in phosphate-buffered 1% osmium tetroxide. Following post-fixation, samples were rinsed for 1 min in filtered distilled water, and were subsequently dehydrated through a graded ethanol series 10 100%. Samples were then given three rinses of propylene and were infiltrated overnight in a 1:1 mixture of propylene oxide:Poly/bed 812 resin. Samples were transferred to pure resin for 4 hr, and were then allowed to harden in a 60°C oven for 3 days. Thin sections were cut on a MT 6000 (Sorvall Instruments, Kendro Laboratory Products, Asheville, NC), then post-stained with saturated uranyl acetate and 0.015% lead. Finally, samples were examined with an EM 900 transmission electron microscope (Zeiss, Jena, Germany), using 50 Kv accelerating voltage.

Statistics. Cytokine data for *Mp*-infected NEC and other conditions were compared using repeated measures

analysis of variance (ANOVA) with Bonferroni post-testing. $P < 0.05$ was taken as significant throughout.

Results

TEM of NEC after MP Infection. TEM showed that NEC cultures were successfully infected by *Mp* at the multiplicity of infection used in this experiment. As expected from previous reports, *Mp* attached to epithelial surface via specialized terminal attachment organelles (Figs. 1 and 2). Treatment of *Mp*-infected NEC cultures with erythromycin appeared to eradicate the organism from cell surfaces (Fig. 3). NEC morphology did not differ in any obvious way between Mp-infected cultures and control cultures, nor was significant detachment of cells observed at the time points assayed.

Cytokines in NEC Supernatants. There were no significant differences between *Mp*-infected and media control NEC at any time point for either IL-8 (Fig. 4) or RANTES (Fig. 5). NEC cultures were capable of mounting chemokine responses to appropriate stimulation, as TNF-α induced significant increases in IL-8 release at all time points, and RSV infection was associated with marked increases in released RANTES at the 48- and 72-hr points. Erythromycin treatment had no significant effect on chemokine release by *Mp*-infected NEC. Pretreatment of RSV with UV irradiation inhibited the RSV-induced RANTES responses (data not shown).

To test whether NEC from asthmatic children are differently "responsive" to *Mp* than NEC from nonasthmatic children, the data were compared between cultures derived from these subgroups. Asthmatic children from whom the cells were obtained were all in the mild to moderate category, and none were taking nasal medications at the time of the nasal biopsies. All asthmatic subjects ($n = 6$) and three of five control subjects had positive skin tests to common aeroallergens, including house dust mites, but none had active rhinitis symptoms at the time brushings were obtained.

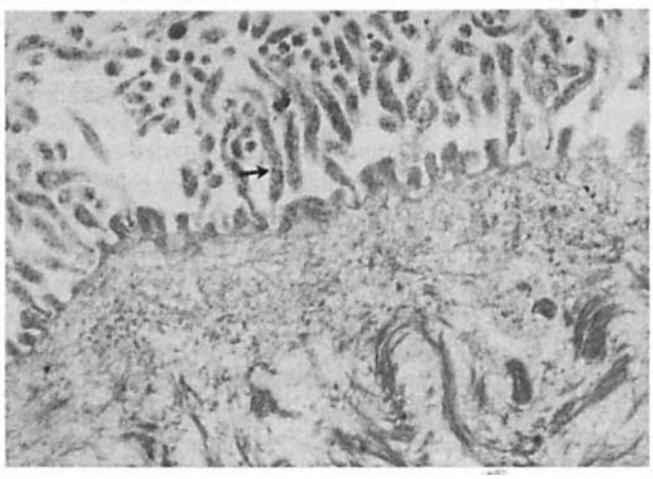

Figure 1. Electron micrograph of epithelial cell surface with adherent *Mp*. Multiple organisms are attached to the surface of the epithelial cell (arrow). Magnification: ×33,600.

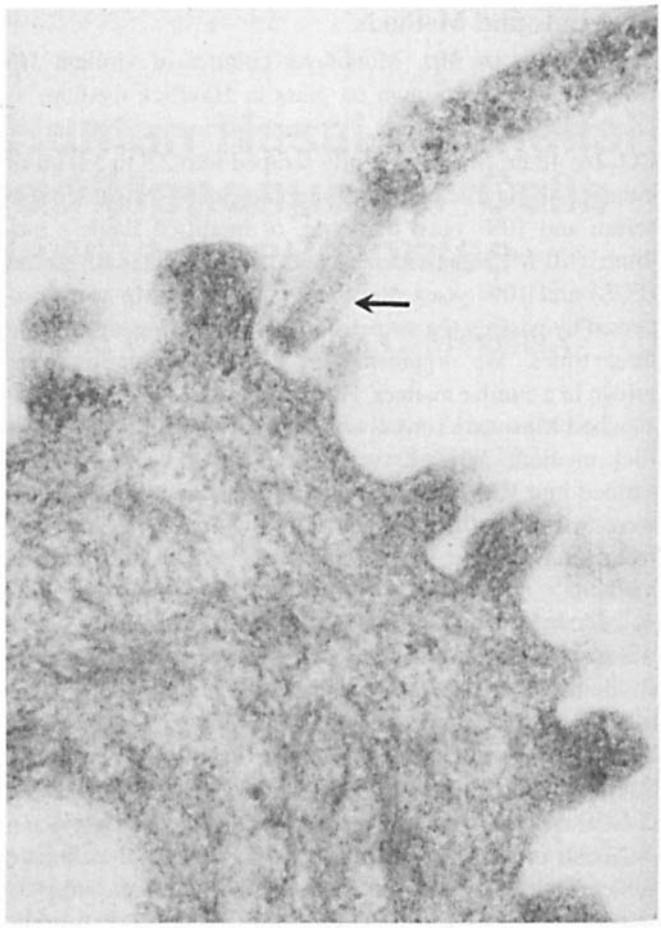

Figure 2. Higher magnification view of epithelial cell surface showing the "tip structure" attachment organelle of *Mp* (arrow). Magnification: ×14,000.

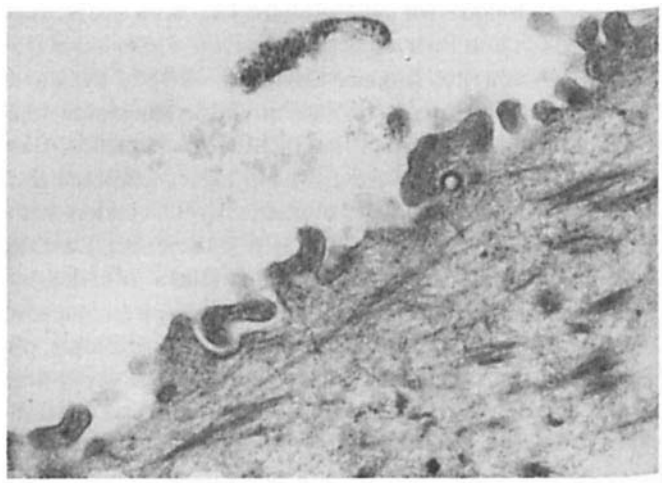

Figure 3. Electron micrograph of epithelial cell surface in culture treated with erythromycin after inoculation with *Mp*. Erythromycin appeared to eradicate organisms from the cell surface. Magnification: ×56,000.

There was no statistically significant difference in chemokine production between asthmatic and nonasthmatic NEC at any of the time points tested, although there was a nonsignificant tendency for IL-8 to be higher among nonasth-

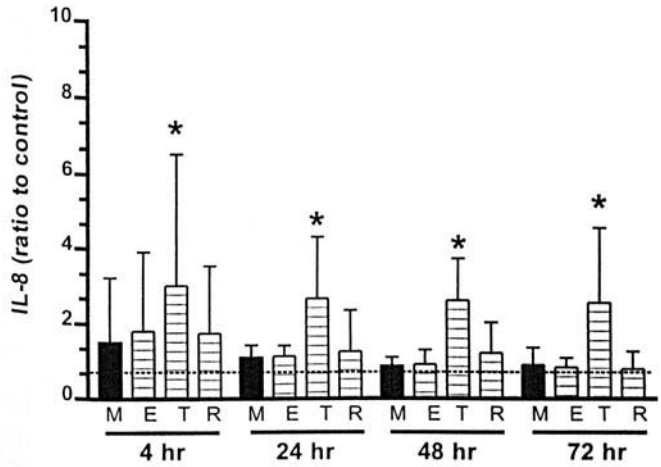

Figure 4. IL-8 release into NEC supernatants at time points after inoculation of NEC cultures (n = 11 experiments). Bars represent mean ± SD for the calculated ratio of each condition to control cultures inoculated with cell culture media alone; horizontal broken line indicates control level (=1). M, *Mp;* E, *Mp* + erythromycin; T, TNF-α; R, RSV. An asterisk indicates $P < 0.05$ vs. control, repeated-measures ANOVA with Bonferroni post-testing.

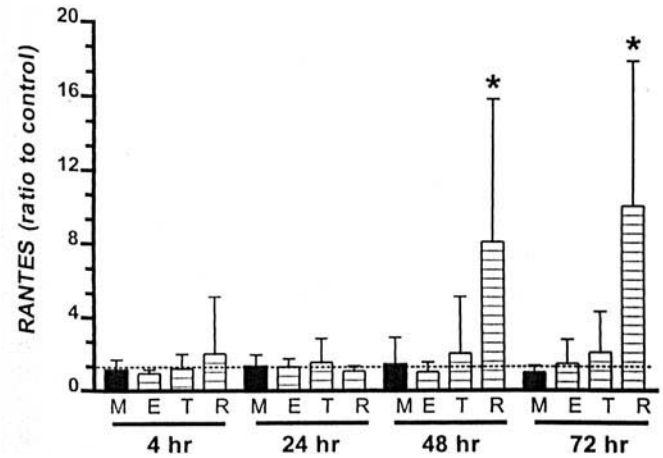

Figure 5. RANTES release into NEC supernatants at time points after inoculation of NEC cultures (n = 11 experiments). Bars represent mean ± SD for the calculated ratio of each condition to control cultures inoculated with cell culture media alone; horizontal broken line indicates control level (=1). M, *Mp;* E, *Mp* + erythromycin; T, TNF-α; R, RSV. An asterisk indicates $P < 0.05$ vs. control, repeated-measures ANOVA with Bonferroni post-testing.

matic NEC (Fig. 6), and for RANTES to be higher among asthmatic NEC early after *Mp* inoculation (Fig. 7).

Cytokines in PBMC Supernatants. Cytokine responses to *Mp* were tested using PBMC from four separate healthy, young adult volunteers. Levels of TNF-α (Fig. 8), IL-2 (Fig. 9), and IL-6 (Fig. 10) were all significantly increased at either 24 or 48 hr after inoculation of PBMC with *Mp*. The *Mp*-stimulated cytokine increases appeared to be of the same order of magnitude as those for LPS.

Discussion

We hypothesized that *Mp* would directly induce production and release of pro-inflammatory cytokines, thereby contributing to airway inflammation in a manner similar to

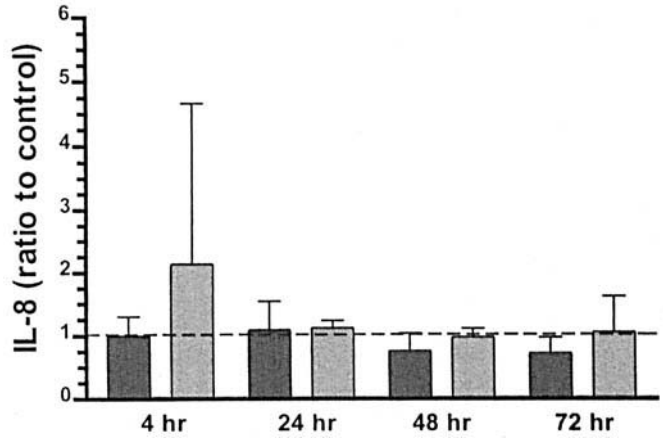

Figure 6. Comparison of IL-8 release by *Mp*-infected NEC derived from asthmatic children (black bars; n = 6) versus nonasthmatic children (gray bars; n = 5). Bars represent mean ± SD for the calculated ratio of each condition to control cultures inoculated with cell culture media alone; horizontal broken line indicates control level (=1). Differences between the two groups were not statistically significant.

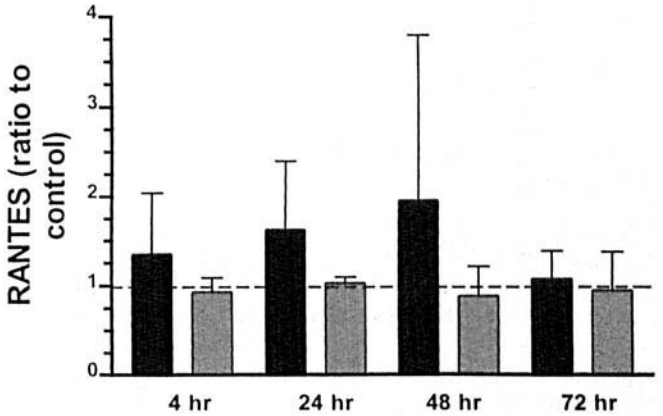

Figure 7. Comparison of RANTES release by *Mp*-infected NEC derived from asthmatic children (black bars; n = 6) versus nonasthmatic children (gray bars; n = 5). Bars represent mean ± SD for the calculated ratio of each condition to control cultures inoculated with cell culture media alone; horizontal broken line indicates control level (=1). Differences between the two groups were not statistically significant.

viral respiratory agents such as RSV. However, in this model we failed to observe significant increases in IL-8 or RANTES production by primary cultures of NEC. This was not due to inability of the NEC culture model to mount a chemokine response, because both TNF-α and RSV produced expected increases (Figs. 4 and 5). Furthermore, there was clear evidence of attachment of abundant *Mp* organisms at the electron microscopy level (Figs. 1 and 2). Our TEM studies showed that *Mp* attached to NEC via terminal attachment organelles, as was previously observed in hamster tracheal organ culture (20). Erythromycin, which inhibits synthesis of Mp attachment proteins (21), appeared to eradicate the organisms (Fig. 3). Finally, the same preparation of *Mp* at the same multiplicity of infection strongly induced release of cytokines by PBMC in a similar pattern to that previously reported by Kita *et al.* (18) (Figs. 8–10).

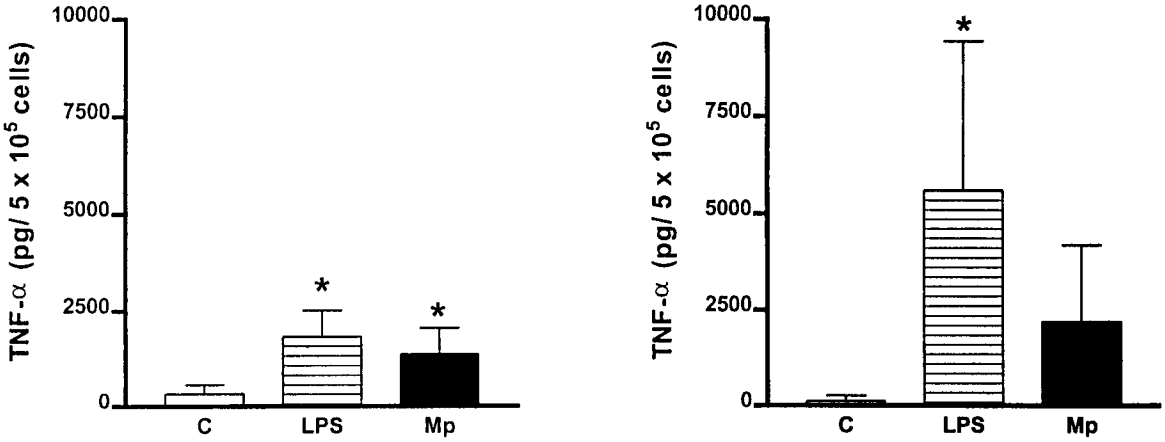

Figure 8. Release of TNF-α by human PBMC at 24 (left) and 48 hr (right) after a 2-hr incubation with control media (C), lipopolysaccharide (LPS), or *Mp* (Mp). Bars represent mean ± SD for $n = 4$ separate experiments. An asterisk indicates $P < 0.05$ vs. control, repeated-measures ANOVA with Bonferroni post-testing.

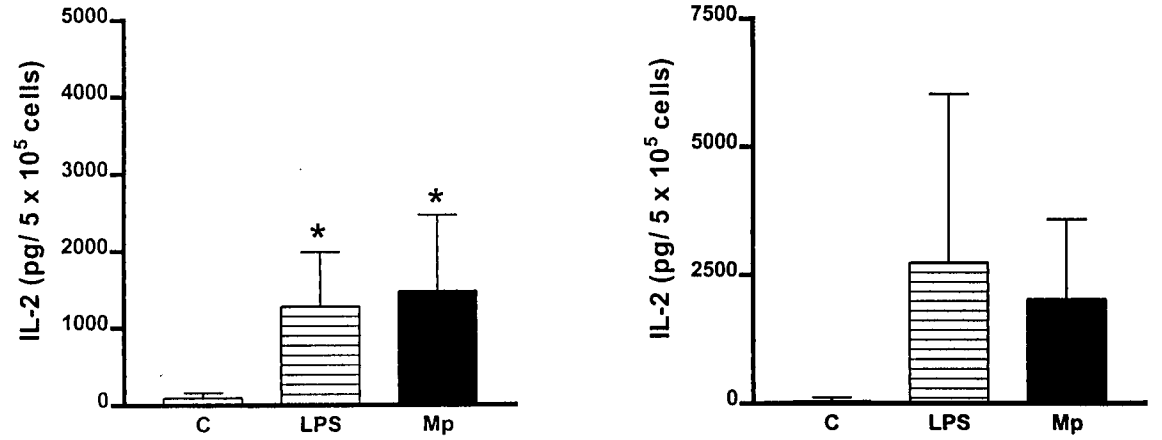

Figure 9. Release of IL-2 by human PBMC at 24 (left) and 48 hr (right) after a 2-hr incubation with control media (C), lipopolysaccharide (LPS), or *M. pneumoniae* (Mp). Bars represent mean ± SD for $n = 4$ separate experiments. An asterisk indicates $P < 0.05$ versus control, repeated-measures ANOVA with Bonferroni post-testing.

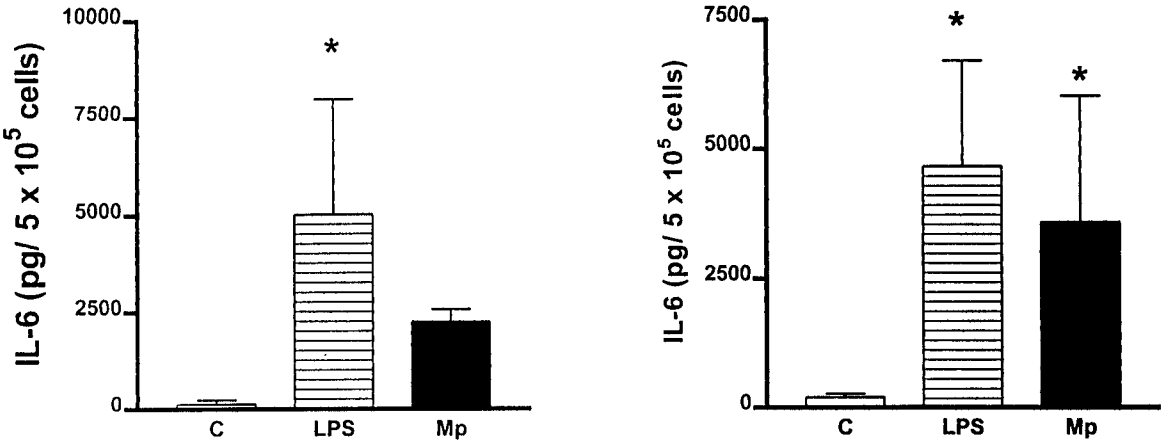

Figure 10. Release of IL-6 by human PBMC at 24 (left) and 48 hr (right) after a 2-hr incubation with control media (C), lipopolysaccharide (LPS), or *M. pneumoniae* (Mp). Bars represent mean ± SD for $n = 4$ separate experiments. An asterisk indicates $P < 0.05$ versus control, repeated-measures ANOVA with Bonferroni post-testing.

There are several potential caveats regarding the lack of *Mp* effect in our experiments. Our NEC studies focused on the release of IL-8 and RANTES, chemokines that are known to be important in the recruitment of inflammatory cells to the respiratory tract, and that have been shown to be upregulated during viral infection of respiratory epithelium in multiple studies. It is possible that *Mp* induces other epithelial mediators or pathways not explored in our studies.

Another caveat is that NEC, although closely resembling large airway cells in humans in terms of both histology and function (23), may not be representative of small airways cells that may be targeted by Mp or other respiratory pathogens. Because we used a single concentration of *Mp* based on previous experience (20–22), it is possible that the "wrong" amount of *Mp* was used to elicit a response. However, the TEM results and the results with PBMC make it unlikely that the multiplicity of infection was too low to stimulate the cells, and NEC showed no morphologic changes suggestive of toxicity. Despite these caveats, the contrasting effects of *Mp* and RSV in our studies suggest that these pathogens may initiate local cytokine amplification through differing mechanisms.

Infection of the respiratory tract with *Mp* is common and may be associated with exacerbations of asthma in adults and children (12–14). Additionally, this pathogen may play a role in maintenance of the asthmatic state. In this regard, *Mp* was detected by PCR in the respiratory secretions of 10 of 18 stable adult asthmatics and in only 1 of 11 healthy controls (13). In another study, throat cultures for *Mp* were positive in 24.7% of children and adults with asthma exacerbation, compared with 5.7% of healthy controls, again suggesting that *Mp* may be an important trigger for asthma attacks (14).

We observed no significant differences between the responses of NEC from asthmatic versus nonasthmatic individuals to *Mp* infection (Figs. 6 and 7). However, the airways of asthmatics are known to be chronically infiltrated with mononuclear cells such as lymphocytes, as well as with eosinophils. We observed strong direct stimulation of normal human PBMC by *Mp* to release a variety of cytokines (Figs. 8–10). This suggests that if similar cell types are already present in the airway at onset of infection, they might amplify airway inflammation via a pathway not present in the nonasthmatic airway.

1. Chapman RS, Henderson FW, Clyde WA, Collier AM, Denny FW. The epidemiology of tracheobronchitis in pediatric patients. Am J Epidemiol **114:**786–797, 1981.
2. Sakurai N, Nagayama Y, Honda A, Makuta M, Yamamoto K, Kojima S. *Mycoplasma pneumoniae* and other pathogens in the aetiology of lower respiratory tract infections among Japanese children. J Infect **16:**253–261, 1988.
3. Berkovich S, Millian SJ, Snyder RD. The association of viral and mycoplasma infections with recurrence of wheezing in the asthmatic child. Ann Allergy **28:**43–49, 1970.
4. Seggev JS, Lis I, Siman-Tov R, Gutman R, Abu-Samara H, Scey G, Naot Y. *Mycoplasma pneumonia* is a frequent cause of exacerbation of bronchial asthma in adults. Ann Allergy **57:**263–265, 1986.
5. Becker S, Reed W, Henderson F, Noah T. RSV infection of human airway epithelial cells causes production of the β-chemokine RANTES. Am J Physiol **272:**L512–L520, 1997.
6. Lampinen M, Rak S, Venge P. The role of interleukin-5, interleukin-8 and RANTES in the chemotactic attraction of eosinophils to the allergic lung. Clin Exp Allergy **29:**314–322, 1999.
7. Folkard SG, Westwick J, Millar AB. Production of interleukin-8, RANTES, and MCP-1 in intrinsic and extrinsic asthmatics. Eur Resp J **10:**2097–2104, 1997.
8. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. Br Med J **16:**982–986, 1993.
9. Grunberg K, Sterk PJ. Rhinovirus infections: induction and modulation of airways inflammation in asthma. Clin Exp Allergy **29S2:**65–73, 1999.
10. Kruger T, Baier J. Induction of neutrophil chemoattractant cytokines by *Mycoplasma hominis* in alveolar type II cells. Infect Immun **65:**5131–5136, 1997.
11. Black HR, Yankaskas JR, Johnson LG, Noah TL. Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor-α and respiratory syncytial virus stimulation. Am J Resp Cell Mol Biol **19:**210–215, 1998.
12. Leaver R, Weinberg EG. Is *Mycoplasma pneumoniae* a precipitating factor in acute severe asthma in children? S Afr Med J **68:**78–79, 1985.
13. Kraft M, Cassel GH, Henson JE, Watsin H, Williamson J, Marmion BP, Gaydos CA, Martin RJ. Detection of *Mycoplasma pneumoniae* in the airways of adults with chronic asthma. Am J Resp Crit Care Med **158:**998–1001, 1998.
14. Gil JC, Cedillo RL, Mayagoitia BG, Paz MD. Isolation of *Mycoplasma pneumoniae* from asthmatic patients. Ann Allergy **70:**23–25, 1993.
15. Pietsch K, Ehlers S, Jacobs E. Cytokine gene expression in the lungs of BALB/c mice during primary and secondary intranasal infection with *Mycoplasma pneumoniae.* Microbiology **140:**2043–2048, 1994.
16. Suga M, Ando M, Mishikawa H, Araki S. Adenosine deaminase activity and free IL-2 receptor levels in serum from patients with *Mycoplasma pneumoniae.* Jpn J Med **30:**108–112, 1991.
17. Ito S, Abe Y, Kinomoto K, Saitoh T, Kato T, Kohli Y, Kuriyama M, Sakai T, Ishizaki T. Fulminant *Mycoplasma pneumonaie* pneumonia with marked elevation of serum soluble interleukin-2 receptor. Intern Med **34:**430–435, 1995.
18. Kita M, Ohmoto Y, Hirai Y, Yamaguchi N, Imanishi J. Induction of cytokines in human peripheral blood mononuclear cells by mycoplasmas. Microbiol Immunol **5:**507–516, 1992.
19. McDonald DM. Upregulation of tachykinin receptors in chronic airway inflammation. Pulmon Pharmacol **8:**203–205, 1995.
20. Muse KE, Powell DA, Collier AM. *Mycoplasma pneumoniae* in hamster tracheal organ culture studied by scanning electron microscopy. Infect Immun **13:**229–237, 1976.
21. Hu PC, Collier AM, Baseman JB. Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. J Exp Med **145:**1328–1343, 1977.
22. Powell DA, Hu PC, Wilson M, Collier AM, Baseman JB. Attachment of *Mycoplasma pneumoniae* to respiratory epithelium. Infect Immun **13:**959–966, 1976.
23. Knowles MR, Carson JL, Collier AM, Gatzy JT, Boucher RC. Measurement of transepithelial electric potential differences in normal human subjects *in vivo.* Am Rev Resp Dis **124:**484–490, 1981.