

***Mycoplasma pneumoniae*–induced activation and cytokine production in rodent mast cells**

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Background: *Mycoplasma pneumoniae* is a respiratory tract pathogen that has been associated with severe exacerbations in patients with chronic asthma. Murine models of infection have recently been established, with disease manifestations similar to those observed in human subjects. Previous studies have suggested that this organism is capable of producing activation of a wide range of immunologic cell types.

Objective: We sought to determine whether *M pneumoniae* can induce mast cell activation in the rodent mast cell line RBL-2H3.

Results: After 4 hours of coculture, morphologic changes indicative of activation were observed by means of electron microscopy, and *M pneumoniae* was identified, by means of immunoelectron microscopy, adhering to mast cell membranes. Coculture of rat basophilic leukemia cells with viable *M pneumoniae* for 4 hours resulted in net release of β -hexosaminidase and serotonin into the supernatant. Live, but not heat-killed, organisms induced the release of IL-4 protein into the culture supernatant, with a peak at 4 hours. During coculture with *M pneumoniae*, production of mRNA for IL-4, IL-6, and TNF- α was upregulated after 2 hours and had returned to near baseline by 24 hours after infection.

Conclusions: We conclude that viable *M pneumoniae* induces activation of mast cells with release of granule contents, as well as cytokine production. (*J Allergy Clin Immunol* 2002;109:470-6.)

Key words: *Mycoplasma pneumoniae*, asthma, mast cells, cytokines

Mycoplasma pneumoniae is a human pathogen that typically infects ciliated epithelial cells in the respiratory tract, producing upper and lower respiratory tract infections. Common clinical manifestations of *M pneumoniae* infection include tracheobronchitis and pharyngitis, but infection can also result in primary atypical pneumonia.^{1,2} The organism has been implicated as a precipitating factor for the development of asthma.³⁻⁵ It has been isolated at a greater percentage from asthmatic patients than from normal nonasthmatic control subjects and has

Abbreviations used

AHR: Airway hyperresponsiveness
 β -hex: β -D-hexosaminidase
RBL: Rat basophilic leukemia

been associated with asthma exacerbations.⁶⁻¹¹ Two recent studies have revealed the presence of *M pneumoniae* DNA in the lower respiratory tracts of about 50% of patients with chronic asthma, suggesting a role for this organism in a subset of patients.^{7,8}

Recently, several groups have established murine models of *M pneumoniae* infection that lead to clinical manifestations of pneumonia similar to those observed in human subjects: pulmonary inflammation, as evidenced by histology; airway hyperresponsiveness (AHR), as measured by means of whole-body unrestrained plethysmography; proinflammatory cytokine mRNA upregulation and protein release into bronchoalveolar lavage; and production of antibodies to *M pneumoniae*.¹²⁻¹⁶ In these studies *M pneumoniae* was detected by means of culture, PCR, or both throughout the entire study, suggesting that long-term pulmonary colonization occurred after nasal installation; the organism could be detected even after symptoms abated. This observation is similar to that seen in human infections with this pathogen, in which *M pneumoniae* can be detected months after the clinical pneumonia has resolved.¹⁴

Although *M pneumoniae* infection usually occurs in the respiratory tract, its target cells are not limited to the epithelium. A number of case reports have documented the presence of this organism in blood, brain, skin lesions, cerebrospinal fluid, the urogenital tract, and the synovium of arthritic joints.¹⁷⁻²³ These observations suggest that *M pneumoniae* may be able to escape the respiratory epithelium and infect cells within the subepithelium and parenchyma of the lung. Also, *M pneumoniae* has been shown to nonspecifically activate cells of the immune system, including B and T cells.^{24,25} *M pneumoniae* infection has been shown to upregulate the gene expression and production of cytokines, including IFN- α , IFN- γ , IL-1 β , IL-2, IL-4, IL-6, and TNF- α in cultured human lymphocytes in vitro.^{26,27}

Because *M pneumoniae* has been isolated from extrapulmonary sites in vivo and has been shown to interact with cells of the immune system in vitro, we hypothesized that the organism could activate mast cells. There is little documentation of the direct effects of mycoplasmas on

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mast cells. Chan et al^{28,29} have reported that *Mycoplasma hyorhinis* infection upregulates the expression of FcεRI and leads to degradation of IgE bound to FcεRI on rat basophilic leukemia (RBL) cells. Wasiela and Brzezinska-Blaszczak³⁰ have documented that *Mycoplasma hominis* stimulates rat peritoneal mast cells to release histamine in vitro. In the present study we investigated the in vitro effects of *M pneumoniae* infection on mast cells. The organism exhibited an ability to activate rat mast cells by inducing the exocytosis of granule contents, as well as by inducing the synthesis and release of cytokines.

METHODS

Cell culture

A clinical isolate of *M pneumoniae* (#19294) was grown in polystyrene tissue-culture flasks to log phase in SP-4 media.² The adherent organisms were harvested with a cell scraper (Costar, Cambridge, Mass), pooled with those floating in the media, centrifuged at 11,000g for 15 minutes, washed twice with PBS, and stored in PBS supplemented with 20% FCS at -70°C in single-use aliquots. Some aliquots of *M pneumoniae* were heat killed by preincubation at 60°C for 30 minutes.

The RBL-2H3 cell line, a rat mucosa mast cell tumor line, was a gift from Dr Robert Hohman (National Institute of Allergy and Infectious Diseases). Cells were propagated in RPMI-1640 medium supplemented with 10% FCS (Hyclone), 100 U/mL penicillin and streptomycin (Cellgro), and 2 mmol/L L-glutamine (Cellgro). For assay, 350,000 cells/mL were plated at 1 mL/well in 24-well plates and then were incubated overnight to confluency (approximately 1×10^6 cells/well). Medium (0.9 mL/well) was replaced before infection.

For infection, 100-μL aliquots of freshly thawed *M pneumoniae* (10^5 to 10^8 CFU/mL final concentrations) were added to each well of RBL cells. The same ratio of organisms to media and cells was maintained if smaller or larger culture conditions were used. Control cells were treated with 100-μL aliquots of 20% FCS in PBS. The length of coculture varied with each experiment.

IgE receptor-mediated activation was used as a positive control. The reagent used consisted of oligomeric rat myeloma IgE (3 μg/mL final concentration) covalently cross-linked as previously described.³¹

Electron microscopy

RBL cells were infected with *M pneumoniae* in 60-mm polystyrene petri dishes for specified incubation periods. In some experiments the organism was immunolabeled for positive identification. Cells were prefixed with 0.1% glutaraldehyde and 3% paraformaldehyde, blocked with 1% BSA and 10% normal goat serum, and labeled with a rabbit polyclonal antibody to *M pneumoniae* (1:100), followed by a 10-nm gold-conjugated goat anti-rabbit antibody (Accurate Chemical, 1:50). In all experiments cells were postfixed with 1% glutaraldehyde, dehydrated through a graded series of alcohols, infiltrated, and embedded in Polybed 812 resin. Sections were poststained with uranyl acetate and lead citrate and viewed at 75 kv on a Hitachi 7000 transmission electron microscope.

Secretion of N-acetyl β-D-hexosaminidase from RBL cells

Adherent RBL cells in a 24-well plate were washed 3 times with RPMI medium without phenol red supplemented with 1% BSA. After replacement with the same medium, *M pneumoniae* was added to each well. After incubation, supernatants were removed, and the cells were lysed with 1 mL of PBS containing 1% Triton X-100. Aliquots (20 μL) of each supernatant or lysate sample were

transferred to a 96-well ELISA plate containing 150 μL of 4 mmol/L pNp-GlcNAc/40 mmol/L citrate/phosphate buffer (pH 4.5) per well. The plate was incubated at 37°C for 1 hour, and the reaction was stopped by the addition of 50 μL of 320 mmol/L glycine/200 mmol/L carbonate buffer (pH 10.0). The plate was read immediately at 410 nm on a Dynatech MRX ELISA reader. The percentage of release of β-D-hexosaminidase (β-hex) was calculated according to the following formula:

$$\text{Supernatant OD}/(\text{Supernatant OD} + \text{Lysate OD}) \times 100.$$

Net percentage of release was calculated by subtracting the percentage of release obtained with buffer alone.

Secretion of tritiated serotonin from RBL cells

Freshly plated RBL cells in a 24-well plate were pulsed with 0.5 μL of tritiated serotonin (1 mCi/mL hydroxytryptamine binoxalate, NET 398, NEN Dupont) per well, and the plate was incubated overnight. Adherent RBL cells were washed 3 times with 0.5 mL/well, followed by the addition of 0.9 mL/well of RPMI, and infected with *M pneumoniae*. After incubation, supernatants were removed, and the unreleased serotonin was collected by lysing the cells with 1 mL of PBS containing 1% Triton X-100. Aqueous supernatants and lysates were counted, and the percentage of serotonin release was calculated according to the following formula:

$$\text{Supernatant cpm}/(\text{Supernatant cpm} + \text{Lysate cpm}) \times 100.$$

Net percentage of release was calculated by subtracting the percentage of release with buffer alone.

Quantitation of cytokine secretion by RBL cells

Supernatants from cultured cells infected with *M pneumoniae* were removed and filtered to remove cellular debris. Cells were lysed with PBS containing 1% Triton X-100. Cell lysates and supernatants were stored at -70°C until assayed. IL-4 protein in the culture supernatant or cell lysate was assessed by using a rat cytokine ELISA (Endogen), according to the manufacturer's instructions.

Detection of cytokine mRNA production in RBL cells

The production of cytokine mRNA by RBL cells during coculture with *M pneumoniae* was assessed with the multiprobe RNase protection assay system (PharMingen), according to the manufacturer's instructions. Briefly, cells were infected with mycoplasma for various time points, supernatants were removed, and RNA was isolated from the adherent cells with TRIzol reagent (Gibco). RNA was hybridized with phosphorous 32-labeled probe overnight, treated with RNase to purify protected probes, and resolved by means of electrophoresis. Bands were visualized by means of autoradiography.

Statistical analysis

Statistical significance was determined with the Student *t* test.

RESULTS

M pneumoniae infection of RBL mast cells

To study the effects of this organism on mast cells in vitro, we infected RBL cells with *M pneumoniae* (10^8 CFU/mL final concentration). We were able to isolate the organism from RBL cell cultures 4 weeks after infection through serial passages, suggesting that the organism is able to successfully colonize this cell type for long periods of time. Coculture with *M pneumoniae* did not result

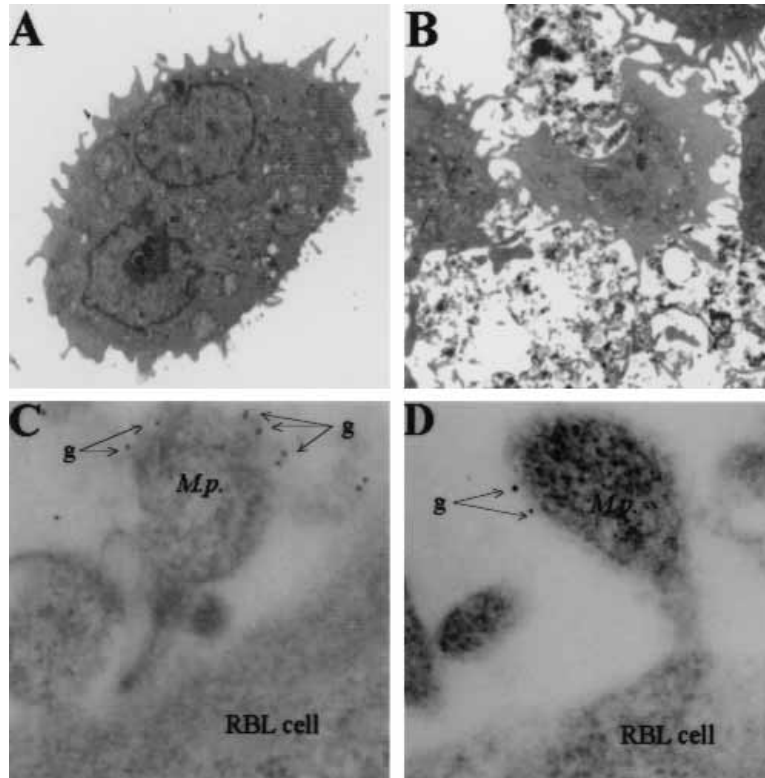


FIG 1. Electron microscopy of RBL cells infected with *M pneumoniae*: **A**, control (noninfected) RBL cell (original magnification 4500 $\times$); **B**, activated RBL cell after a 4-hour coculture with 10^8 CFU/mL *M pneumoniae* (original magnification 5000 $\times$); **C** and **D**, *M pneumoniae* attached to RBL cell membranes after a 4-hour coculture with 10^8 CFU/mL *M pneumoniae* (**C**, original magnification 40,000 $\times$; **D**, original magnification 50,000 $\times$). Mycoplasmas in **C** and **D** were immunolabeled with 10-nm gold beads (*g*) for positive identification.

in increased RBL cell death, as measured by trypan exclusion over a 72-hour period, the longest infection period in subsequent experiments.

After 4 hours of coculture with the organism, morphologic changes in RBL cells indicative of activation (ie, membrane ruffling and exocytosis of granules) were observed by means of electron microscopy (Fig 1, *B*). No such changes were noted in control cells (Fig 1, *A*) or in cells incubated with heat-killed *M pneumoniae* (data not shown). Visualization of the organism interacting with RBL cells was observed by means of immunoelectron microscopy (Fig 1, *C* and *D*).

RBL cell degranulation caused by *M pneumoniae* infection

Because there was morphologic evidence of RBL cell activation after coculture with *M pneumoniae*, we determined whether the organism could induce the release of inflammatory mediators. After a 4-hour coculture with live, but not heat-killed, organisms, β -hex was released from RBL cells in a dose-dependent manner (Fig 2, *A*). Similar results were obtained when considering both β -hex and serotonin net release ($6.3\% \pm 1.9\%$ and $8.4\% \pm 0.9\%$ [mean $\pm$ SD], respectively) after coculture with 10^8

CFU/mL *M pneumoniae* (Fig 2, *B*). The net amount of both β -hex and serotonin released after infection with the organism was approximately one tenth the net release after maximal IgE-mediated stimulation (β -hex, $57.0\% \pm 5.8\%$; serotonin, $57.9\% \pm 1.9\%$; data not shown). A time-course study revealed that an increase in the net release of β -hex occurred within the first 2 hours after introduction of the organism into the culture ($6.9\% \pm 2.1\%$; Fig 2, *C*). On replacement of the culture media after 2 hours of coculture, no subsequent significant increase in net release of β -hex occurred.

M pneumoniae-induced cytokine release from RBL cells

After establishing that *M pneumoniae* induced the release of inflammatory mediators from RBL cells, we were interested in determining whether cytokines were also released. After a 4-hour coculture with live, but not heat-killed, organisms, IL-4 (69.1 ± 50.5 pg/mL) was released into the culture supernatant (Fig 3, *A*). As with β -hex release, the amount of IL-4 released by *M pneumoniae* was approximately one tenth of the amount released after maximal IgE-mediated stimulation (776.2 ± 258.6 pg/mL, data not shown).

Interestingly, when considering the time course of IL-4 release after *M pneumoniae* infection, the cytokine was undetectable in the culture supernatant at 2 hours after infection, was near maximal levels at 4 hours after infection (34.5 ± 19.9 pg/mL), and peaked at 6 hours after infection (38.2 ± 22.3 pg/mL; Fig 3, B). There was a kinetic difference in the time course of IL-4 release after *M pneumoniae* infection compared with that after IgE-mediated stimulation. After treatment with oligomeric IgE, IL-4 was detected at 2 hours after treatment (157.3 ± 147.5 pg/mL) and peaked at 4 hours after treatment (422.9 ± 323.2 pg/mL; Fig 3, C).

The differences between the release patterns of β -hex (Fig 2, C) and IL-4 (Fig 3, B) from RBL cells after *M pneumoniae* infection suggests that unlike β -hex, which is stored in granules and is available for release immediately on activation, cytokine release depends entirely on the generation of newly synthesized protein. We therefore looked for the presence of IL-4 protein in the lysates of unstimulated control cells to determine whether protein was stored. IL-4 protein was not detectable in control cell lysates. IL-4 protein was detected in cell lysates after either a 4-hour stimulation with IgE (257.8 ± 121.1 pg/mL) or a 4-hour coculture with 10^8 CFU/mL *M pneumoniae* (83.3 ± 46.1 pg/mL, data not shown).

Cytokine mRNA production after coculture with *M pneumoniae*

Once it was established that *M pneumoniae* induced the release of IL-4 from RBL cells, we wanted to determine the cytokine mRNA profile produced after coculture with the organism. Results are shown in Fig 4. Control cells (noninfected RBL cells) demonstrated basal activities for IL-4, IL-6, and TNF- α (Fig 4, lane 1). After coculture with *M pneumoniae* (10^8 CFU/mL) for 2, 4, and 6 hours (Fig 4, lanes 3, 5, and 7, respectively), these species of mRNA were upregulated. The same pattern of cytokine mRNA upregulation was seen after treatment with IgE (data not shown). Also upregulated were small amounts of IL-3 after a 2-hour infection (Fig 4, lane 3) and IL-5 after a 6-hour infection (Fig 4, lane 7) with 10^8 CFU/mL *M pneumoniae*. These values had returned nearly to baseline by 24 hours after infection. Infection with a low dose of *M pneumoniae* (10^5 CFU/mL) did not induce the upregulation of cytokine mRNA at any of the time points (Fig 4, lanes 2, 4, 6, and 8). As noted in the release assays, live, but not heat-killed, organisms induced the upregulation of cytokine mRNA (data not shown). Cytokine mRNA that was not upregulated in this assay included IL-1, IL-2, IL-10, TNF- β , and IFN- γ .

DISCUSSION

As a respiratory tract pathogen, *M pneumoniae* has been implicated in the pathogenesis of asthma. Infection with *M pneumoniae* has been observed, in some cases, to precede the development of asthma symptoms; previously nonasthmatic individuals displayed increased AHR, decreased FEV₁, and subsequent wheezing after *M pneu-*

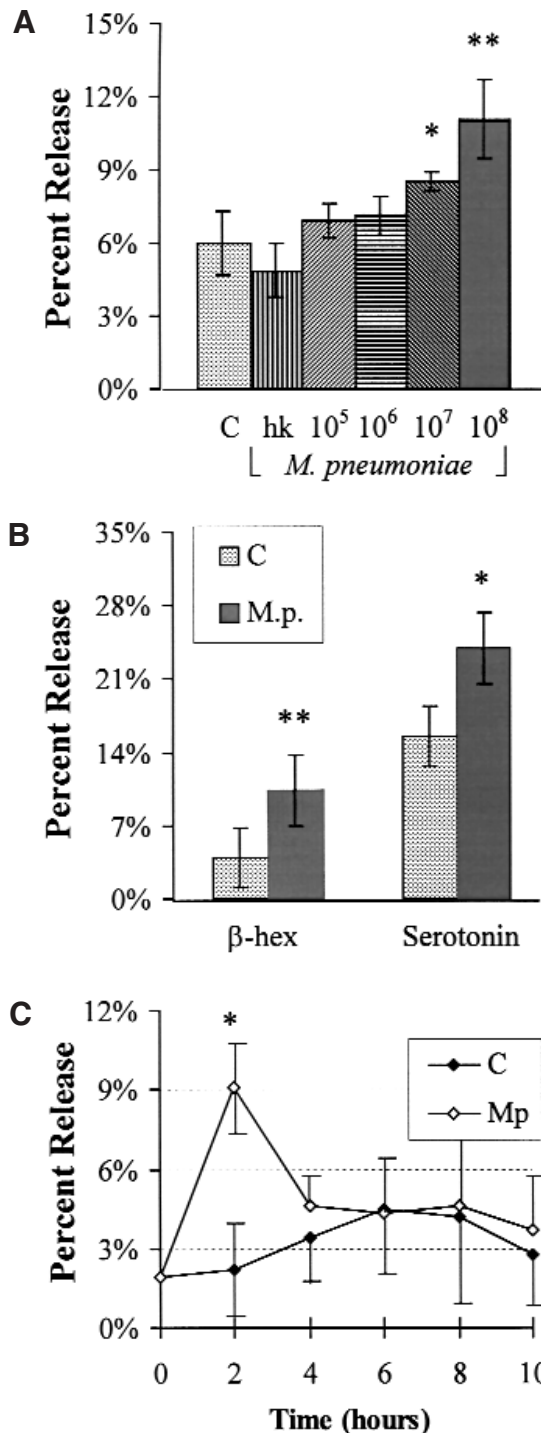


FIG 2. Inflammatory mediators released from RBL cells after *M pneumoniae* infection: **A**, dose response of β -hex release after a 4-hour coculture with heat-killed (hk) or live *M pneumoniae* (10^5 – 10^8 CFU/mL; $n = 3$ experiments; $*P = .033$, $**P = .013$; **B**, release of β -hex ($n = 9$ experiments, $**P < .001$) and serotonin ($n = 5$ experiments, $*P = .009$) after a 4-hour coculture with 10^8 CFU/mL *M pneumoniae*; **C**, time course of β -hex release after 10^8 CFU/mL *M pneumoniae* infection ($n = 3$ experiments, $*P = .002$). Degranulation, as measured by the release of β -hex into the culture supernatant, was quantitated spectrophotometrically. Data are expressed as mean $\pm$ SD release.

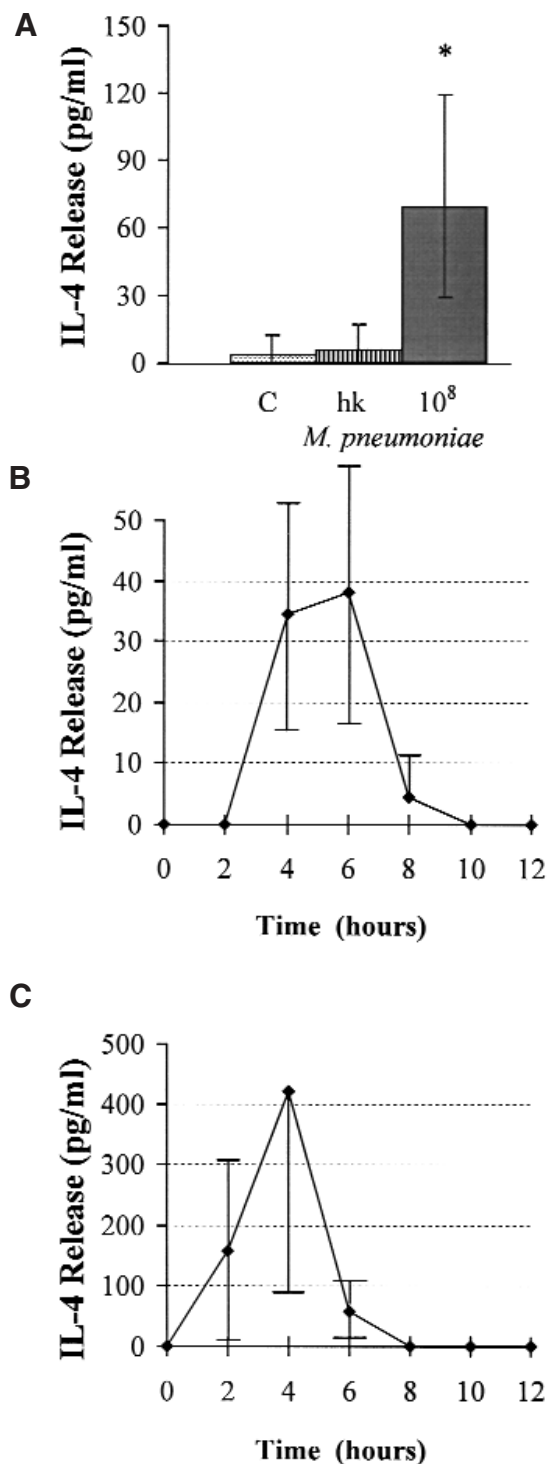


FIG 3. Release of IL-4 from RBL cells after addition of heat-killed (hk) or live *M pneumoniae* (10^8 CFU/mL): **A**, release of IL-4 after a 4-hour coculture with *M pneumoniae* ($n = 12$ experiments, $*P < .001$ vs C); **B**, time course of IL-4 release after coculture with 10^8 CFU/mL *M pneumoniae* ($n = 3$ experiments); **C**, time course of IL-4 release after treatment with 3 μ g/mL oligomeric IgE ($n = 3$ experiments). The amount of IL-4 protein released into culture supernatants was determined by using ELISA. Data are expressed as means $\pm$ SD.

moniae infection.^{3,5} The organism has been isolated from a greater proportion of asthmatic subjects than nonasthmatic control subjects, suggesting that asthmatic or allergic individuals may be more susceptible to chronic colonization by this organism.⁶⁻⁸ Increased levels of IgG₁ and IgE specific for *M pneumoniae* have been shown in patients after infection.³² The presence of *M pneumoniae*-specific IgE offers the potential for mast cell involvement in the immune response after infection by this organism. Interestingly, Martin et al⁸ have reported that chronic asthmatic subjects who had positive PCR results for *M pneumoniae* had greater numbers of infiltrating mast cells in bronchial tissue samples than asthmatic subjects with negative PCR results.

Mast cells have been shown to play an important role in the pathogenesis of asthma. In murine models of asthma, mast cell-deficient animals failed to develop AHR after antigen sensitization and challenge, suggesting an important role for this cell type in the development of asthma.^{33,34} Furthermore, mast cell-derived mediators, such as histamine, leukotrienes, prostaglandins, and cytokines, have been well established as triggers for asthmatic exacerbations.³⁵ In human nasal and bronchial biopsy specimens, mast cell numbers in the epithelium, as well as the relative amounts of immunoreactive IL-4, IL-5, IL-6, and TNF- α in those mast cells, were increased in allergic or asthmatic subjects compared with levels in control subjects.³⁶⁻³⁸

Because mast cells play a fundamental role in asthma, direct activation of mast cells by *M pneumoniae* offers a potential role for this organism in the pathogenesis of this disease. In the present study we demonstrated that *M pneumoniae* was able to activate rodent mast cells in vitro. RBL cells exhibited morphologic changes indicative of activation after coculture with *M pneumoniae*. Coculture with the organism also induced the release of inflammatory mediators, the release of IL-4, and the upregulation of mRNA for proinflammatory cytokines. This activation was dependent on live organisms; heat-killed organisms did not elicit the same responses.

The importance of IL-4 in allergic airway responses has been documented. Elevated IL-4 and IL-4R α mRNA and protein levels have been detected in the asthmatic airway, and mast cells have been shown to express both proteins.³⁸⁻⁴¹ In murine models of asthma, neutralization of IL-4 with antibodies administered during antigen sensitization prevented the development of allergic airway eosinophilia and AHR.^{42,43} Attenuation of asthmatic responses occurred in IL-4-deficient animals, which, compared with control animals, displayed significantly fewer eosinophils in bronchoalveolar lavage and lung tissue, reduced airway inflammation, reduced AHR, reduced IL-4 and IL-5 production, and no development of ovalbumin-specific IgE in response to antigen sensitization and challenge.⁴³⁻⁴⁵ These responses were completely abrogated in IL-4R α - and STAT6-deficient mice.⁴⁶⁻⁴⁹ Furthermore, STAT6-deficient mice failed to have a T_H2 response after antigen sensitization and challenge, suggesting a critical role for IL4R α and STAT6 signaling in the development of the T_H2 phenotype.⁴⁹

Because IL-4 is considered to be one of the central cytokines involved in asthma, *M pneumoniae*-induced synthesis and release of IL-4 from mast cells provides a possible link between infection by this organism and the development, exacerbation, or both of asthma. In this study we have demonstrated the synthesis and release of IL-4 after coculture with *M pneumoniae*. Because the population of cells responsible for generation of the initial burst of IL-4 in allergic airway responses has not been definitively proven, release of IL-4 from mast cells after *M pneumoniae* infection could be important as the initiator of the cascade leading to T_H2 phenotype commitment, IgE production by B cells, inflammatory cell infiltration into the lung epithelium, and the subsequent development of AHR.

An interesting observation in this study was that the pattern of cytokine mRNA generated in RBL cells after *M pneumoniae* infection (Fig 4) was virtually identical to the pattern generated after IgE-mediated stimulation (data not shown). This suggests that *M pneumoniae* may be interacting in some way with FcεRI on the surface of mast cells. *M pneumoniae* attachment to host cells most likely occurs through interactions between adhesion proteins localized to the specialized terminal structure on the bacterium and host glycoprotein sialic acid residues.⁵⁰⁻⁵² FcεRI on rat mast cells is a glycoprotein, and N-linked carbohydrate chains present on the extracellular portion of the receptor α-chain have been shown to possess terminal sialic acid residues, offering the potential for the interaction between *M pneumoniae* and this receptor.^{53,54}

In summary, the results of this study demonstrate the ability of *M pneumoniae* to directly activate rat mast cells in vitro. The ability of *M pneumoniae* to induce mast cell degranulation, as well as the synthesis and release of IL-4 from mast cells, offers a potential link between infection by the organism and the pathogenesis of asthma.

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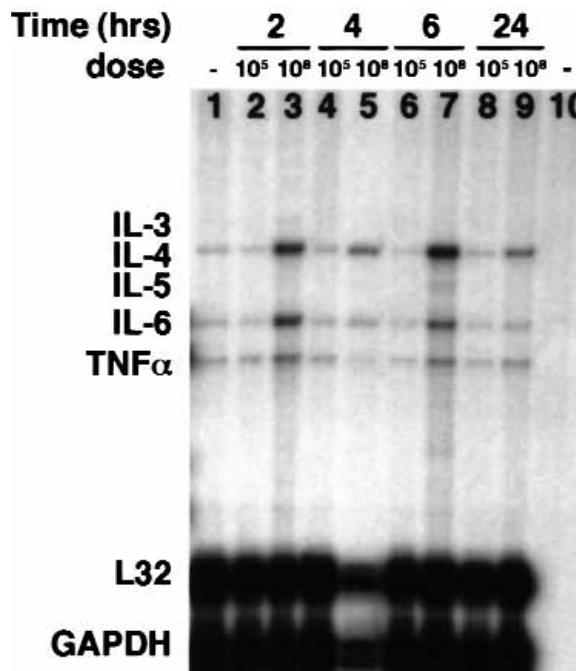


FIG 4. *M pneumoniae*-induced cytokine mRNA upregulation in RBL cells. RNase protection assay showing mRNA production by RBL cells after coculture with *M pneumoniae* for the indicated times and at the indicated concentrations. mRNA for IL-4, IL-6, and TNF-α was upregulated after coculture with the high dose of *M pneumoniae* only. Note: Lane 5 was loaded unevenly. Representative results from 6 experiments are shown.

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