

Isotype-specific antibody responses to acute *Mycoplasma pneumoniae* infection

Joram S Seggev, MD;*† Gerald V Sedmak, PhD‡; and Viswanath P Kurup, PhD†

Background: *Mycoplasma pneumoniae*-induced respiratory infections affect millions of patients and have been implicated in exacerbation of bronchial asthma. IgE may be involved in such exacerbations. While specific IgG and IgM responses to *Mycoplasma pneumoniae* are well described, the response of other isotypes is less known.

Purpose: To determine whether specific IgE and what subclasses of IgG are formed in response to *Mycoplasma pneumoniae* infection.

Methods: We studied 20 patients with acute *Mycoplasma pneumoniae* infection, in whom the diagnosis was confirmed by a 16-fold increase in complement fixation titer between acute and convalescent serum samples. We developed an enzyme-linked immunosorbent assay (ELISA) for the determination of IgG, IgA, and IgM antibodies specific for *Mycoplasma pneumoniae* protein antigens. We used Western blotting to confirm the results of the ELISA and to detect Mycoplasma-specific IgG subclasses and IgE.

Results: Changes in *Mycoplasma pneumoniae*-specific IgG, IgA, and IgM were significant. Western blots of *Mycoplasma pneumoniae* antigens in 13 convalescent sera showed specific IgG in all, IgM in 11, IgA in 6, and IgE in 10. The IgG response consisted mainly of IgG1 and IgG3, and to a lesser degree of IgG2 and IgG4.

Conclusions: We conclude that *Mycoplasma pneumoniae* infection is associated with a significant specific IgA and IgE response, in addition to the well-known responses of IgG and IgM. As IgE is involved in allergic reactions, the production of *Mycoplasma pneumoniae*-specific IgE may have a role in exacerbation of bronchial asthma.

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INTRODUCTION

Mycoplasma pneumoniae causes about 20,000,000 infections in Americans per year, of which 500,000 are pneumonia, 11,500,000 are other respiratory infections, and the remainder are probably asymptomatic infections.^{1,2} *Mycoplasma pneumoniae* infection may have long lasting effects on lung

function, even in normal subjects. Such infections may be associated with exacerbation of bronchial asthma, which affects 6% to 8% of the American population.³⁻⁹

The diagnosis of *Mycoplasma pneumoniae* infection is difficult, because the bacterium may take up to 8 weeks to grow in culture, which has a sensitivity of 57%.¹⁰ The widely used complement fixation test is limited by the inability to measure isotypes other than IgG. The glycolipid antigen employed in the complement fixation assay cross reacts with multiple unrelated substances; protein antigens of *Mycoplasma pneumoniae* are less likely to cross react with unrelated antigens.¹⁰⁻¹³

Most patients develop *Mycoplasma pneumoniae*-specific IgG and IgM.^{10-12,14,15} *Mycoplasma pneu-*

moniae-specific IgA develops in serum and in respiratory secretions but the response of this isotype has been studied less than IgG or IgM.¹⁵⁻¹⁶ Changes in total IgE have been reported during *Mycoplasma pneumoniae* infections, but few data on serum Mycoplasma-specific IgE are currently available.^{9,17,18} Because IgE has a role in the exacerbation of asthma, changes in IgE during *Mycoplasma pneumoniae* infections may be important.

In the present study we determined class-specific antibodies, including IgE, to *Mycoplasma pneumoniae* protein antigens in patients with acute respiratory infections. Our results indicate enhanced *Mycoplasma pneumoniae*-specific IgA and IgE responses in these patients.

MATERIALS AND METHODS

Sera, Patients, and Controls

Serum samples had originally been obtained from hospitalized patients by their primary physicians for confirmation of the diagnosis of *Mycoplasma pneumoniae* or viral (for control) pneumonia. After being found to be positive for *Mycoplasma pneumoniae* or respiratory viruses by complement fixation, the remainders of the samples were referred to us. One ambulatory patient with confirmed *Mycoplasma pneumoniae* bronchitis was included following the signing of informed consent. The mean ($\pm$ standard deviation, SD) age of the 6 male and 13 female subjects was 25.3 ± 17.1 (range 2 to 59) years. One patient was asthmatic and one had a family history of asthma. *Mycoplasma pneumoniae*-specific complement fixation titers were <8 in acute sera and ranged from 1:128 to 1:4096 (median 1:256) in convalescent sera. Four patients had serologic evidence of concomitant viral in-

* University of Nevada School of Medicine, Las Vegas, Nevada, and † Clement J Zablocki Veterans Affairs Medical Center and Medical College of Wisconsin, Milwaukee, Wisconsin, and ‡ City of Milwaukee Health Department Laboratory, Milwaukee, Wisconsin.

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fections, respiratory syncytial virus in two and one each with influenza and parainfluenza type 3. Control sera were obtained from 11 patients who had positive complement fixation tests for respiratory viral infections, and negative (<8) for *Mycoplasma pneumoniae* complement fixing antibody. They included eight females and three males, whose mean age was 45.4 ± 27.4 (range 9 to 85) years. Ten had influenza A and B (one with concomitant evidence for adenovirus) and one with respiratory syncytial virus infection. Additional control sera were obtained from 78 male and 17 female healthy faculty, staff, and students of the University of Missouri-Columbia whose mean age was 30 ± 5.6 years. Five *Mycoplasma pneumoniae*-positive sera with complement fixation titers ranging from 1:256 to 1:1024 were obtained from the Centers for Disease Control as positive controls for this part of the study. The study was approved by the Institutional Review Boards of the University of Nevada, The Medical College of Wisconsin and Zablocki Veterans Affairs Medical Center, and of the University of Missouri-Columbia and Harry S. Truman Memorial Veterans Hospital, Columbia, MO.

Reagents

Frozen pellets of washed *Mycoplasma pneumoniae* strain PI-1428 in phosphate-buffered saline (PBS) were purchased from Bionique Laboratories, Saranac Lake, NY. Chemical reagents were purchased from Sigma Chemical Company, St. Louis, MO; Kirkegaard & Perry Laboratories, Gaithersburg, MD, and Pierce Chemical Company, Rockford, IL; protein G (suspension of 10% Streptococci G) from Zymed Laboratories, South San Francisco, CA; horseradish peroxidase-streptavidin and avidin; unconjugated, horseradish peroxidase-conjugated and biotin-conjugated goat anti-human IgE, IgA and IgM; mouse anti-human IgG Fc; goat anti-mouse IgG; and rabbit anti-goat IgG Fc from Sigma, Vector (Burlingame, CA), Kirkegaard and Perry, Jackson Immuno-research

(West Grove, PA), and Zymed. All polyclonal antisera were affinity-purified. The following monoclonal anti-human antibodies were purchased from Hybridoma Research Laboratory, Kingsville, MD; anti-IgG₁ HP 6069, anti-IgG₂ HP 6002+6014, anti-IgG₃ HP 6047+6050, and anti-IgG₄ HP 6025.

Assays

Complement fixation titers for *Mycoplasma pneumoniae* and respiratory viruses (influenza types 1–3, parainfluenza, adenovirus, and respiratory syncytial virus) were performed by employing commercially available reagents (Whittaker) at the Virology Laboratory of the City of Milwaukee Health Department.

Preparation of antigen for ELISA

Washed *Mycoplasma pneumoniae* were lysed with Triton X-114 in tris buffer containing phenyl-methyl-sulfonyl-fluoride for 30 minutes at 4°C .¹⁹ After centrifugation at 12,000 g, the supernatant was warmed, leading to gelation of the triton phase, trapping lipids. Following another centrifugation at 3000 rpm, the supernatant contained the aqueous protein mixture that was used as antigen. Protein concentration was determined with bis-phenol-chromic acid (BCA kit, Pierce Chemicals Co). An aliquot was taken for sodium dodecyl phosphate polyacrylamide gel electrophoresis (SDS-PAGE) for quality control. The characterization of the mixture of proteins employed Western blotting with rabbit-anti-*Mycoplasma pneumoniae* and monoclonal anti-P1, the major adhesion protein of pathogenic *Mycoplasma pneumoniae* (Fig 1).^{20,21} These assays were performed by Joel Baseman, PhD, Department of Microbiology, University of Texas Health Sciences Center, San Antonio.

ELISA for *Mycoplasma pneumoniae*-Specific IgG, IgM and IgA

Immulon 2 (Dynatech) plates were coated with *Mycoplasma* antigen, $4\mu\text{g}/\text{mL}$, in carbonate-bicarbonate buffer, pH 9.6.²² After blocking with bovine serum protein (BSA) and washing with

phosphate buffered saline (PBS)-Tween 20 0.05%, the following were added in succession: appropriately diluted serum (incubated for 75 minutes, 37°C); horseradish peroxidase-conjugated isotype-specific antiserum (incubated for 90 minutes, 37°C); and substrate, tetramethyl benzidine (incubated for 15 minutes, room temperature). Optical density (OD) was determined on an automated ELISA plate reader at a wavelength of 630 nm. For *Mycoplasma pneumoniae*-specific IgM determinations, sera were pre-absorbed with protein G in order to remove IgG.²³

Immunoblotting of *Mycoplasma pneumoniae* Protein Antigens with Human Sera

Frozen *Mycoplasma pneumoniae* were thawed, diluted in PBS, and boiled in reducing sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 7% to 15% polyacrylamide gradient gel and the proteins were transferred to nitrocellulose, which was cut into strips and blocked.^{24,25} One strip each with molecular weight markers and *Mycoplasma pneumoniae* antigenic proteins were stained with colloidal gold to ensure successful transfer of proteins.²⁶ Other nitrocellulose strips were incubated with diluted serum overnight at 4°C (1:100 for IgG, IgM, and IgA; 1:50 for IgG subclasses; 1:20 for IgE). For IgG, IgA, IgM, and IgE, biotinylated isotype-specific antisera were added, followed by horseradish peroxidase-avidin D. Color was developed with 4-chloro-1-naphthol or tetramethyl benzidine. In some experiments, *Mycoplasma pneumoniae*-specific IgE was detected by a double-antibody technique, employing goat anti-human IgE followed by biotinylated rabbit anti-goat IgG and horseradish peroxidase-streptavidin, a modification of an assay for Aspergillus-specific IgE.²⁶ For IgG subclasses determination, a subclass-specific monoclonal antibody was followed by horseradish peroxidase-conjugated goat anti-mouse IgG. In every experiment, controls without serum were set up to ascertain speci-

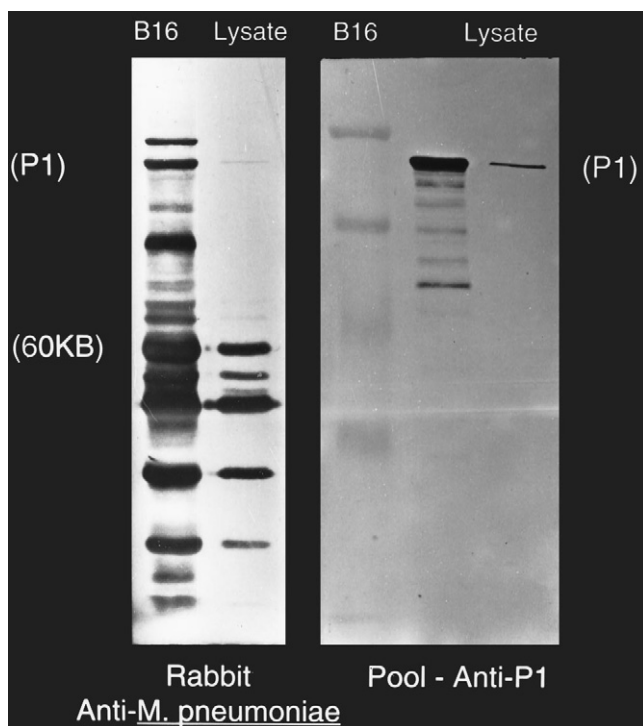


Figure 1. Western blot of whole *Mycoplasma pneumoniae* strain B16 and Triton X-114 extract of strain PI-1428, which used as antigen for ELISA. Left panel, rabbit and anti-whole *Mycoplasma pneumoniae*. Right panel, pooled monoclonal anti-P1.

ficity. In some experiments, sera from control patients with acute viral respiratory diseases were included as negative controls.

Statistics

For statistical evaluation, we used Wilcoxon's rank-sum two sample tests for comparisons of paired data and Fish-

er's exact test for comparison of categorical data. Correlations were determined with Spearman's correlation test.

RESULTS

Our ELISA results correlated well ($0.85, P < .005$) with complement fixation, as the patients had a significant specific IgG response to *Mycoplasma pneumoniae* (Table 1). Fourteen of 17 patients showed a change of $\geq 50\%$ in OD of specific IgG between the acute and convalescent specimens, which were obtained a mean of 18 days (range 4 to 30 days, median 16 days) after the acute specimen. Three sera had initial complement fixation titers $>1:256$ and were included in the convalescent sera. There was a marked IgA response in 12 out of 13 patients studied. Another two adults had a high IgA OD and complement fixation titer $>1:256$ on the initial serum specimen, and therefore were included among convalescent sera in Table 1. Of eight adult patients, seven had $\geq$ fourfold increases in OD (range ≤ 0.3 acute to ≤ 1.78 convalescent) and two initial high OD, whereas four children under the age of 8 years and one 23-year-old patient, OD values doubled, but remained below 0.6. One patient had no IgA response, but demonstrated strong

Table 1. *Mycoplasma pneumoniae*-Specific Antibody by ELISA*

Iso-type	Serum dilution	Acute†		Convalescent†		PS‡
		No	OD	No	OD	
Patients with Mycoplasma infection						
IgG	200	18	0.58 ± 0.28	17	1.01 ± 0.43	<.002
IgA	100	13	0.36 ± 0.26	15	0.82 ± 0.44	<.002
IgM	200	18	0.51 ± 0.35	20	0.69 ± 0.36	.0235
Patients with viral respiratory disease						
IgG	200	8	0.51 ± 0.23	11	0.81 ± 0.33	.052
IgA	100	8	0.33 ± 0.18	11	0.46 ± 0.18	.1
IgM	200	8	0.48 ± 0.26	11	0.44 ± 0.32	.71
Healthy subjects						
IgG	200	95	0.56 ± 0.27			
IgA	100	95	0.23 ± 0.12			
IgM	200	95	0.31 ± 0.14			

* Expressed as optical density at 630 nm (mean $\pm$ SD).

† Complement fixation titer for *Mycoplasma pneumoniae* <8 .

‡ Complement fixation titer for *Mycoplasma pneumoniae* 128–4096.

§ Statistical evaluation by Wilcoxon's rank-sum 2 sample test.

|| Complement fixation titer positive for influenza A and B, adenovirus, and respiratory syncytial virus; negative for *Mycoplasma*.

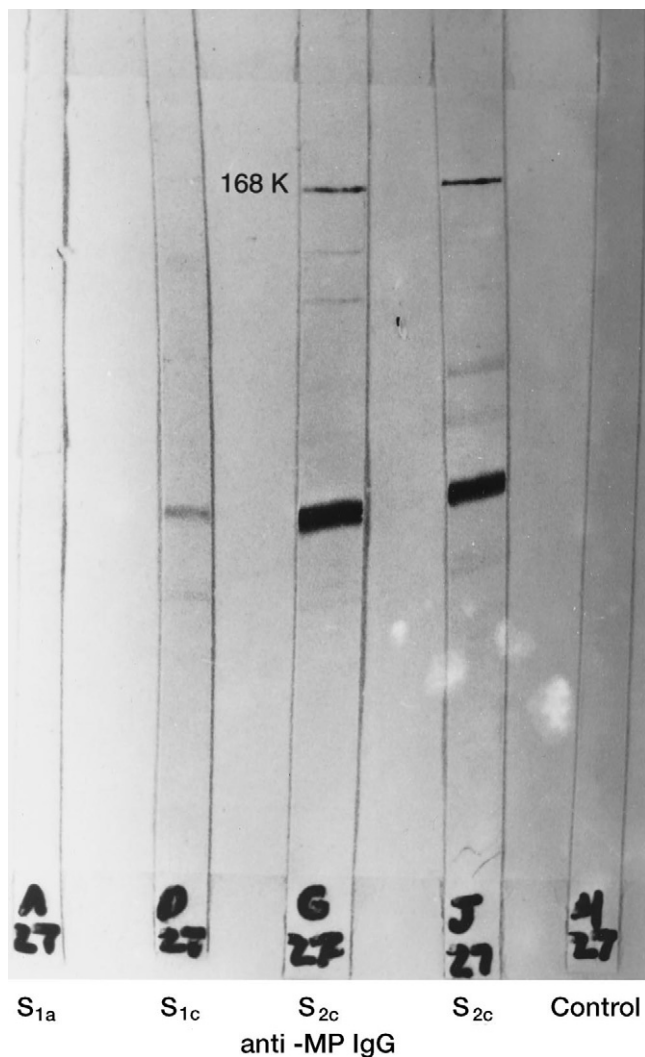


Figure 2. Western blot of acute (a) and convalescent (c) sera of 2 representative patients (S1 and S2) for *Mycoplasma pneumoniae*-specific IgG. The control blot has no patient's serum. 168k is the band compatible with P1.

IgG and IgM responses. The specific IgG and IgA responses showed a weak correlation (0.58). The initial OD of *Mycoplasma pneumoniae*-specific IgM of these patients' sera was above the 75th percentile values in 95 healthy controls (Table 1), and showed a small, but significant increase in convalescent samples. Nine of 12 patients had a significant IgM response. Sera from respiratory virus-infected patients showed a barely-significant IgG response by ELISA, but no significant IgM or IgA responses.

On Western blotting, acute samples showed weak or no bands, with an

occasional strong band at 60 kD, which was seen in some healthy subjects as well. Convalescent sera showed several bands with antigens ranging from about 60 to 200 kD (Fig 2–3, showing representative blots for IgG and IgA). A faint band could be seen in a few acute sera at a point corresponding to P1 (168kD), the adhesion protein and major immunogen of pathogenic *Mycoplasma pneumoniae*, where a significantly stronger band was seen in convalescent sera. The IgG response was associated primarily with IgG₁ and IgG₃, whereas IgG₂ and IgG₄ showed weak bands in

half the subjects tested (Table 2). The number of patients developing *Mycoplasma pneumoniae*-specific IgA by Western blotting was smaller than the number developing specific IgM ($P = .054$), as sera with a low OD of *Mycoplasma pneumoniae*-specific IgA on ELISA showed no bands on blotting.

Mycoplasma pneumoniae-specific IgE developed in 10 of 11 patients tested, reacting mainly with P1 (Fig 4), regardless of a personal or family history of allergy. Heat inactivation of serum at 56 °C for 30 minutes resulted in loss of bands on Western blots. In one patient followed prospectively, *Mycoplasma pneumoniae*-specific IgE could be detected up to 2 months from initial symptoms, but not at 5 months.

Sera from patients with viral diseases showed no specific bands, although they occasionally showed staining at 60 kD, which was seen in healthy subjects as well.

DISCUSSION

The current study demonstrates the formation of antibodies of all isotypes in response to *Mycoplasma pneumoniae* infection. The diagnosis of acute infection was confirmed by complement fixation, which has a sensitivity and specificity of >90%.¹⁰ We did not use the complement fixation test for the determination of other isotypes because it measures mainly IgG, unless modified.^{14–15} Furthermore, the glycolipid antigen employed in the assay cross reacts with multiple other antigens.^{10,12,27}

Our data are in agreement with other studies, showing that most patients develop *Mycoplasma pneumoniae*-specific IgG and IgM, with IgM titers tending to be higher in younger patients.^{10–12,15} Respiratory tract *Mycoplasma pneumoniae*-specific IgA may be more reliable in the diagnosis than IgM, particularly in adults.^{15,16} In our series of patients, *Mycoplasma pneumoniae*-specific IgA developed in most patients, being more pronounced in older patients, in whom we could demonstrate specific IgA by Western blotting. *Mycoplasma pneumoniae*-specific IgA was the most discriminat-

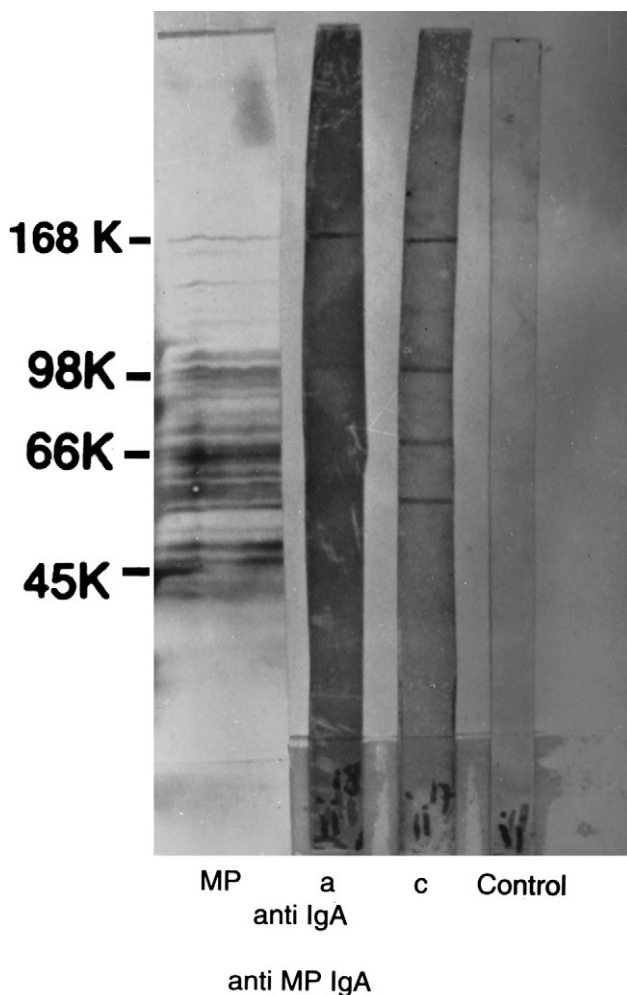


Figure 3. Western blot of acute (a) and convalescent (c) representative serum for *Mycoplasma pneumoniae*-specific IgA, also showing *Mycoplasma pneumoniae* antigen (MP), obtained by SDS-PAGE, blotting into nitrocellulose, and gold-stained. The control blot has no patient's serum.

Table 2. *Mycoplasma pneumoniae*-Specific Antibodies by Immunoblotting

Isotype	Acute*	Convalescent*	With Strong Bands	Control†	
				Acute	Convalescent
IgM	2 /11	11 /12‡	10	0 /8	0 /11
IgA	1 /7	6 /10	2	0 /8	0 /11
IgE	1 /9	10 /11‡	3	0 /8	0 /11
IgG	4 /11	13 /13	10	0 /8	0 /11
IgG ₁	4 /9	10 /10	5	0 /8	0 /11
IgG ₂	3 /9	4 /8	0	0 /8	0 /11
IgG ₃	3 /8	8 /8	8	0 /8	0 /11
IgG ₄	1 /9	4 /7	0	0 /8	0 /11

* No positive/no tested.

† Sera positive for viral respiratory diseases and negative for *Mycoplasma pneumoniae* by complement fixation.

‡ $P = .05$, Fisher's exact test, convalescent versus acute samples.

ing antibody on ELISA. The nearly significant change in *Mycoplasma pneumoniae*-specific IgG in patients with viral infections may be due to polyclonal activation of B lymphocytes, leading to a booster phenomenon and an anamnestic production of IgG antibodies to agents of past infections.¹¹

The pattern of reactivity for IgG and IgM on Western blots in our study was similar to previously published data.^{28,29} The IgG response consisted mainly of IgG₁ and IgG₃, while specific IgG₂ and IgG₄ responses were less common and weaker. The pattern of *Mycoplasma pneumoniae*-specific IgA was similar to that of IgG, supporting the correlation noted on ELISA. The absence of specific bands on Western blotting in the virus-infected patients further increases the validity of our data.

We also show that *Mycoplasma pneumoniae* infection led to the production of specific IgE in all but one of the convalescent sera tested. Specific IgE reacted mainly with P1. The absence of reaction on Western blots with acute sera and the reduction in the intensity of the staining of convalescent sera with heat inactivation suggest that we were able to show a specific IgE response. Nagayama et al reported that total IgE and unrelated allergen-specific IgE increased in *Mycoplasma pneumoniae*-infected children, and decreased during convalescence.¹⁷ Wheezing occurred in asthmatic and atopic patients who had higher IgE.¹⁷ Increased bronchial hyperreactivity in asthmatics and the development of clinical asthma during convalescence from *Mycoplasma pneumoniae* infection have been recently reported. The affected patients had increased total serum IgE and developed *Mycoplasma pneumoniae*-specific IgE.^{9,30} An earlier study reported the detection of *Mycoplasma pneumoniae*-specific IgE in a number of atopic patients, but the time period between possible infection and sampling was not mentioned.¹⁸ An IgE response to infection has also been reported in mice, in which a murine *Mycoplasma*, *M. pulmonis*, led to IgE

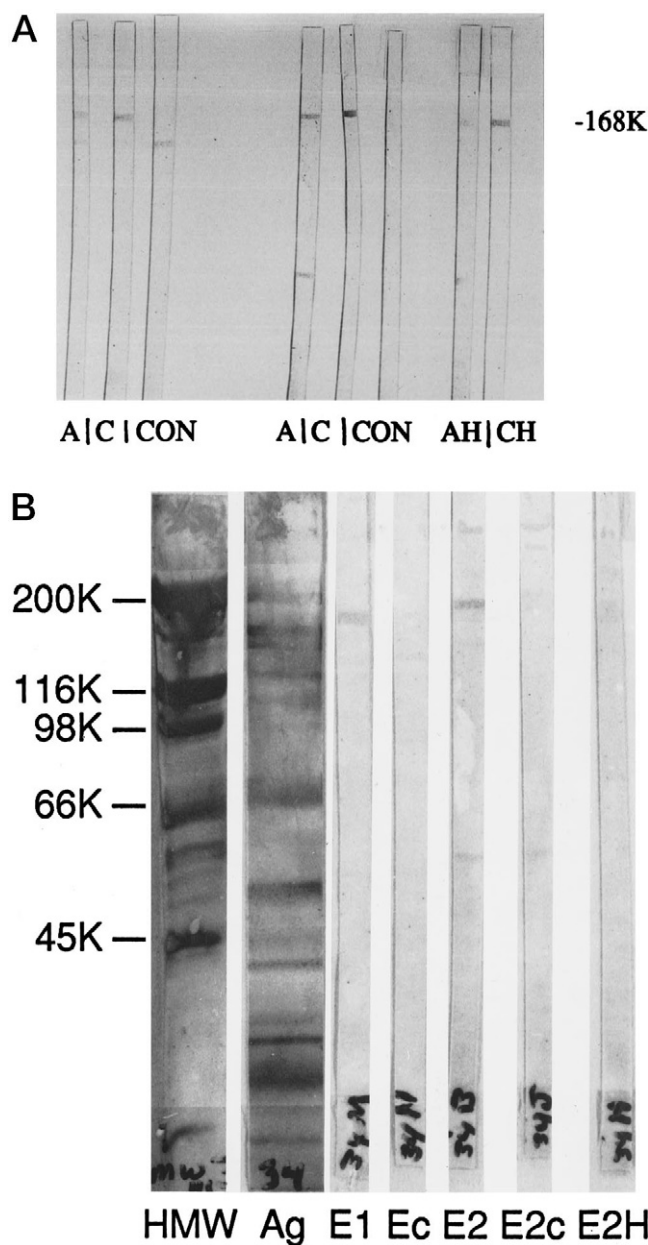


Figure 4A. Western blot of an acute and convalescent (A and C) representative sera for *Mycoplasma pneumoniae*-specific IgE, and a blot with both heat-inactivated sera (AH and CH). The left blots were developed by biotinylated goat anti-human IgE, followed by avidin D-horseradish peroxidase. The center and right hand blots were developed by goat antihuman IgE, followed by biotinylated rabbit anti-goat IgG and streptavidin horseradish peroxidase. The control blots (CON) have no patient's serum. The heat inactivated strip loses (AH) or has reduced intensity (CH) of the band seen at the 168-kD mark. Figure 4B. Western blot of 2 convalescent (E1 and E2) representative sera for *Mycoplasma pneumoniae*-specific IgE, a blot with heat-inactivated serum (E2H), high-molecular weight markers (HMW), and *Mycoplasma pneumoniae* antigen (Ag), obtained by SDS-PAGE, transferring into nitrocellulose, and gold-stained. The control blots (Ec and E2c) have no patient's serum. The heat inactivated strip loses the band seen at the 168-kD mark on strips E1 and E2.

production by splenic and upper respiratory lymph node lymphocytes.³¹ The production of IgE in serum and, possibly, locally in the respiratory tract, may be significant as IgE-mediated inflammation could potentiate the initial inflammatory response caused by *Mycoplasma pneumoniae* in the airways.³²

Mycoplasma pneumoniae has several effects on the immune system and on respiratory epithelium. It activates lymphocytes, leads to polyclonal immunoglobulin synthesis, and causes the release of cytokines such as interferon-alpha and granulocyte-macrophage colony stimulating factor, which were reported to be instrumental in inflammation.³³⁻³⁵ *Mycoplasma pneumoniae* also causes epithelial damage, followed by infiltration with mononuclear cells.¹⁻² The combination of bacterially induced and IgE-mediated inflammation may be additive and may contribute to significant clinical effects on lung function.

In summary, we have shown that there is a significant IgA and IgE response to *Mycoplasma pneumoniae* infection, in addition to a specific IgG response. The production of *Mycoplasma pneumoniae*-specific IgE may be of significance in atopic patients and may be associated with bronchial asthma.

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Request for reprints should be addressed to:
 Joram S. Seggev, MD.
 Associate Professor
 Department of Internal Medicine
 University of Nevada School of Medicine
 2040 West Charleston Blvd. Suite #300
 Las Vegas, Nevada 89102