

Relationship of Pre-Existing Antibody to Subsequent Infection by *Mycoplasma pneumoniae* in Adults

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An extensive study of the epidemiological and serological characteristics of *Mycoplasma pneumoniae* infection was carried out in a military population. There was an increase in the infection rate at Camp Lejeune during the summer months as indicated by a relative increase in isolations, seroconversions, and hospitalizations for *M. pneumoniae* pneumonia. Twenty-three percent of the trainees who later became infected had detectable, pre-existing antilipid antibody to *M. pneumoniae*. When the whole organism was used as antigen, a pre-existing complement fixation (CF) titer of 1:4 or greater correlated with resistance to *M. pneumoniae* disease as defined by the absence of a fourfold rise in CF antibody, shedding of organisms, and clinical illness. Pre-existing antilipid fraction CF antibody titers of 1:16 or greater correlated with protection against mild and severe *M. pneumoniae* disease. Antilipid CF antibody titers of 1:4 and 1:8 were related to protection against mild disease but were not associated with protection against pneumonia which required hospitalization. The severity of illness was directly related to the CF antibody response in trainees with acute respiratory disease and pneumonia due to *M. pneumoniae*. The findings provide a basis for the development of a *M. pneumoniae* vaccine.

Mycoplasma pneumoniae infections continue to account for a significant proportion of disease due to respiratory infections in military trainees (25). A number of serological methods have been developed for detecting *M. pneumoniae* antibody, including immunofluorescence (9, 11, 22, 23), indirect hemagglutination (23), tetrazolium reduction inhibition (19), complement fixation (CF) (7, 10), and the sensitive radioimmunoprecipitation (1) and mycoplasmacidal tests (2). Until now, only antibodies detected by the tetrazolium reduction inhibition (21) and fluorescent antibody (8) techniques have been associated with protection against illness.

At Camp Lejeune, N.C., where *M. pneumoniae* infections are prevalent, we had the unusual opportunity of studying the effect of pre-existing *M. pneumoniae* antibody on the incidence and severity of natural infection, since sera are obtained from all recruits when they enter training. Knowledge about the effect of pre-existing antibody on natural *M. pneumoniae* infections will be even more impor-

tant when killed and live, attenuated *M. pneumoniae* vaccines are developed. For instance, recipients of some inactivated virus vaccines developed unusually severe illness when they were later infected with wild virus (3, 13). In this study we present evidence that high levels of pre-existing antilipid and anti-whole organism CF antibodies were associated with resistance to *M. pneumoniae* infection and disease. Although a large proportion of trainees with intermediate levels of pre-existing antilipid CF antibody did develop pneumonia, a sensitizing effect for intermediate levels of antilipid antibody was not demonstrated conclusively.

MATERIALS AND METHODS

Trainees. The study population has been described (21, 25). Sera were obtained from all Marine Corps trainees entering the training depot at Parris Island, S.C., stored at -20 C, and tested with matching sera obtained at a later date. Trainees spent an initial 8 to 10 weeks at Parris Island, S.C., and were then transferred, without leave, 300 miles to Camp Lejeune, N.C., for additional training.

Camp Lejeune trainees acutely ill with respiratory diseases reported to the dispensary, were examined by a U.S. Navy medical corpsman or a physician, and

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were given therapy or referred for hospitalization at the Naval Hospital, Camp Lejeune, N.C. Charts from all trainees hospitalized during the calendar years 1970 and 1971 were reviewed for this study.

The *M. pneumoniae* surveillance carried out by the Naval Medical Field Research Laboratory included cultures for *M. pneumoniae* obtained on up to 40 trainees per week reporting to the dispensary with acute respiratory disease. All trainees hospitalized for acute respiratory disease were cultured likewise. Acute and convalescent sera (obtained 10 to 21 days after the acute specimens) were collected from approximately 50% of the trainees cultured. The content of this report was derived in part from the results of 6,163 *M. pneumoniae* isolation attempts and 1,646 CF tests carried out by our laboratory over a period of 3 years.

Isolation of *M. pneumoniae*. Throat gargle specimens were obtained in veal infusion broth and inoculated into diphasic medium (16). The diphasic agar consisted of 3.4 g of Difco pleuropneumonia-like organism (PPLO) agar, 60 ml of distilled deionized water, and 0.1 ml of 2% phenol red solution. This mixture was autoclaved at 121 C for 15 min and then allowed to cool to 56 C. A separate solution consisting of 20 ml of gamma globulin-free horse serum (Grand Island Biological), 10 ml of yeast extract (Microbiological Associates), 10 ml of 10% glucose, 3 ml of thallium acetate, 1.0 ml of 0.2% methylene blue, and 1.0 ml of penicillin (100,000 U/ml) was heated to 56 C. The two solutions were combined and dispensed in 1.0-ml volumes into 2-dram (about 2.4 g) vials. Agar butts solidified at room temperature and were stored at 4 C for up to 30 days.

PPLO broth base contained 3.5 g of PPLO broth powder (Difco), 100 ml of distilled deionized water, and 0.17 ml of 2% phenol red, autoclaved at 121 C for 15 min. The diphasic broth contained 60 ml of PPLO broth base, 30 ml of mycoplasma supplement (33% yeast extract, 66% gamma globulin-free horse serum, Difco), 10 ml of 10% glucose, 3 ml of thallium acetate, 1.0 ml of 0.2% methylene blue, and 1.0 ml of penicillin (10,000 U/ml). The diphasic broth was dispensed in 2.0-ml quantities into the vials containing 1.0 ml of diphasic agar. The diphasic agar butt-broth medium was combined only as was necessary to inoculate fresh specimens. Specimens were incubated at 34 C in the diphasic medium for up to 2 months or until a pH change indicated growth of *M. pneumoniae*.

M. pneumoniae organisms were subcultured at 37 C on PPLO agar plates tightly sealed with paraffin. Plates were prepared with the same agar formula used in the diphasic butts except that phenol red, methylene blue, and glucose were absent. All media were tested before use for inhibitory substances against *M. pneumoniae* by incubation with known dilutions of a low-passage wild-type *M. pneumoniae* organism.

M. pneumoniae colonies were presumptively identified if they adsorbed erythrocytes from a 0.5% suspension of fresh guinea pig erythrocytes applied in Alsever solution.

Whole organism CF antigen. *M. pneumoniae* strain FH, originally isolated by Liu (17), was grown on glass in medium similar to that described by Chanock et al. (6). Nine hundred milliliters of solu-

tion was prepared in distilled deionized water and contained 5.0 g of glucose, 7.4 g of PPLO broth powder without crystal violet (CV) (dehydrated, Difco), 4.8 g of Eagle minimal essential medium powder (Grand Island Biological, F-15), 11.9 g of *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES, A grade, Calbiochem). The solution was sterilized by membrane (Millipore Corp.) filtration. PPLO medium (1,000 ml) was prepared by the addition of 50 ml of yeast extract, 50 ml of gamma globulin-free horse serum, 1.25 ml of 2% thallium acetate (Fisher), and 500,000 U of penicillin to the solution. The pH of the medium was adjusted to 7.4 with sodium hydroxide.

Strain FH was stored at -60 C in HEPES broth in 1.0-ml volumes. A 0.5-ml amount of strain FH was inoculated into 40 ml of PPLO medium in 16-oz (about 0.47 liter) Povitsky bottles and incubated horizontally at 37 C until the glass contained a 75 to 90% sheet of PPLO colonies and the pH began to decrease. The medium was then decanted, and attached organisms were washed four times with 10 ml of phosphate-buffered saline (pH 7.3). The organisms were scraped from the glass or removed by freezing and thawing, resuspended in 0.5 ml of distilled water, and pooled. Whole organism CF antigen produced titers of 1:16 to 1:32 against 4 to 8 U of antibody.

Chloroform-extracted *M. pneumoniae* lipid CF antigen (15). Organisms were grown on glass as described above, and the lipids were extracted by shaking with a mixture of chloroform, methanol, and 0.10 N potassium chloride (20:10:7.5). The mixture was allowed to stand for 18 h at 4 C in a separatory funnel. The chloroform fraction was withdrawn and the chloroform was evaporated under a hood. Antigen was suspended in a small amount of phosphate-buffered saline.

CF tests. CF tests were carried out by the micro-titer method of Sever (20), by using 2 exact U of complement and 4 to 8 U of antigen.

Seroepidemiological study of antilipid antibodies. Preinfection, acute, and convalescent sera were obtained from 296 trainees either reporting to the dispensary or hospitalized between August 1970 and February 1971 (Tables 1-3). Fifty-three were controls reporting to the dispensary with non-respiratory complaints. The remaining 243 were 132 trainees reporting to the dispensary with the chief complaint of symptoms of acute respiratory disease, and 111 were trainees hospitalized with a diagnosis of acute respiratory disease or pneumonia. Predominant microbial agents isolated from these trainees by our laboratory or by the laboratory at the Naval Hospital, Camp Lejeune, N.C., included *M. pneumoniae*, adenovirus type 7, and beta hemolytic streptococcus (25). Additional serological studies revealed a small percentage of parainfluenza type 1 and 3 infections (24). No attempt was made to exclude individuals infected with more than one agent from the study. All 888 individual serum specimens were tested for the presence of *M. pneumoniae* antibody by the CF test by using chloroform-extracted *M. pneumoniae* lipids as antigen (Tables 1-3), as well as using the whole organism preparation as antigen (Table 4). For further prospective analysis, sera obtained from 569 additional trainees upon arrival at Parris Island were

TABLE 1. Relationship of level of pre-existing antilipid *M. pneumoniae* complement-fixing antibody to subsequent *M. pneumoniae pneumonia*^a

Pre-existing antilipid antibody titer	<i>M. pneumoniae pneumonia</i> (hospitalized)		<i>M. pneumoniae</i> disease without pneumonia (hospitalized plus out-patients)		No subsequent <i>M. pneumoniae</i> disease ^b	
	No.	%	No.	%	No.	%
1:16 or greater . . .	0 ^c	0	3	5	32	42
1:4 to 1:8	7 ^d	35	9 ^e	15	15	20
Less than 1:4	13	65	50	81	29	38

^a Chi-square tests (Yates' correction) used in Tables 1-4. Fourfold rise in *M. pneumoniae* antibody between the acute and convalescent sera plus pneumonia by roentgenograph.

^b No fourfold rise at any stage of training.
^c Pneumonia versus no subsequent disease, 0.005 > P > 0.001.

^d Pneumonia versus no pneumonia, 0.20 > P > 0.10.

^e No pneumonia versus no subsequent disease, 0.02 < P < 0.05.

TABLE 2. Relationship of intermediate levels of pre-existing *M. pneumoniae* antilipid complement-fixing antibody to subsequent infection by *M. pneumoniae* organisms

Pre-existing antilipid antibody titer	Patients subsequently treated for <i>M. pneumoniae</i> infection ^a						No subsequent <i>M. pneumoniae</i> disease	
	Out-patients only		Hospitalization required		All with disease			
	No.	%	No.	%	No.	%	No.	%
1:4 to 1:8	6 ^b	16	10 ^c	24	16 ^d	20	15	43
Less than 1:4	31	84	32	76	63	80	29	66

^a Fourfold CF rise taken as evidence of infection.
^b Outpatients versus no subsequent disease, 0.20 > P > 0.10.
^c Hospitalized versus no subsequent disease, 0.50 > P > 0.40.
^d All disease versus no subsequent disease, 0.20 > P > 0.10.

included as controls in the CF test results presented in Table 4.

RESULTS

***M. pneumoniae* surveillance.** An increase in the rate of recovery of *M. pneumoniae* occurred each summer during 1969, 1970, and 1971 (Fig.

1). A special *M. pneumoniae* surveillance was carried out during the fall of 1970 and early winter of 1971; sera were obtained from trainees on arrival at Parris Island, and acute and convalescent sera were obtained at Camp Lejeune from the same men. Of 296 trainees studied, 220 (74%) developed a fourfold rise in antilipid antibody between the time of collection of the Parris Island specimen and the time of the second Camp Lejeune specimen. A total of 138 of the trainees seroconverted before the acute Camp Lejeune serum specimen was obtained, and 82 men developed a fourfold rise in *M. pneumoniae* antibody between the acute and convalescent specimens at Camp Lejeune (59% of the seroconversions). Of the 82 men who seroconverted at Camp Lejeune, 45 men re-

TABLE 3. Correlation of high levels of pre-existing *M. pneumoniae* antilipid complement-fixing antibody with resistance to *M. pneumoniae* disease

Pre-existing antilipid antibody titer	Patients subsequently treated for <i>M. pneumoniae</i> infection ^a						No subsequent <i>M. pneumoniae</i> disease	
	Out-patients only		Hospitalization required		All with disease			
	No.	%	No.	%	No.	%	No.	%
1:16 or greater . . .	0 ^b	0	3 ^c	7	3 ^d	4	32	42
Less than 1:16 . . .	37	100	42	93	79	96	44	58

^a Fourfold CF rise taken as evidence of infection.
^b Outpatients versus no subsequent disease, 0.0005 > P.
^c Hospitalized versus no subsequent disease, 0.0005 > P.
^d All disease versus no subsequent disease, 0.005 > P.

TABLE 4. Correlation of pre-existing complement-fixing whole-organism *M. pneumoniae* antibody with resistance to *M. pneumoniae* infection

Preinfection CF titer	Hospitalized trainees (fourfold CF rise)				Acute respiratory disease, <i>M. pneumoniae</i> isolation, and fourfold CF rise		Controls ^a	
	No.	%	No.	%	No.	%		
1:4 or greater	3 ^b	4	3 ^c	6	107	19		
Less than 1:4	71	96	46	94	462	81		

^a Sera obtained at random from 569 trainees on arrival at Parris Island and not selected as to whether they later developed a fourfold CF rise.
^b Hospitalized versus controls, P < 0.005.
^c Acute respiratory disease versus controls, P < 0.05.

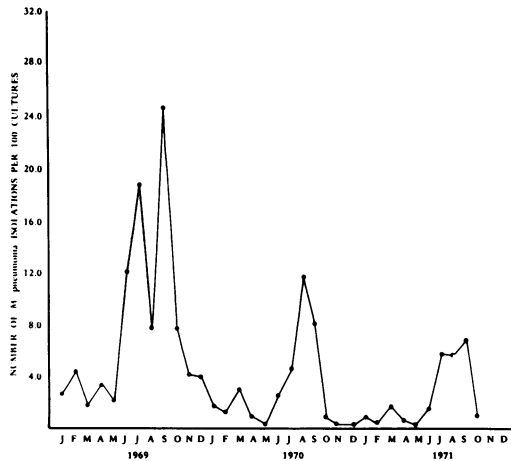


FIG. 1. Number of *M. pneumoniae* isolations per 100 cultures at Camp Lejeune. Note the relative increase in *M. pneumoniae* isolations during the summer months. The relative increase was exaggerated by adenovirus epidemics each winter, which increased the total number of men sampled during the winter as compared with the summer months.

quired hospitalization for acute respiratory disease. Twenty of the hospitalized trainees had pneumonia (Table 1). The additional 37 men were treated as outpatients for acute respiratory disease at Camp Lejeune (Table 2). Therefore, a fourfold rise in *M. pneumoniae* antibody was associated with pneumonia in 24% of trainees studied for acute respiratory disease at Camp Lejeune. Seventy-six of the 296 trainees (26%) failed to develop a fourfold rise in *M. pneumoniae* CF antibody during the 10- to 16-week training period (Table 1).

Seroepidemiological studies were begun in August 1969, and the seasonal peaks in the proportion of *M. pneumoniae* isolations paralleled increases in the proportion of seroconversions (fourfold rises in CF titer) to anti-whole organism antibody (Fig. 2). Note that there was also an increase in the number of patients with *M. pneumoniae* pneumonia hospitalized during the summer months of the years 1970 and 1971 (Fig. 3).

Protective effect associated with pre-existing antibody. CF results discussed in this section apply only to sera obtained on arrival at Parris Island, S.C., and, therefore, before *M. pneumoniae* infection had occurred. The data presented in Tables 1, 3, and 4 indicate that pre-existing antilipid or anti-whole organism CF antibody was associated with protection against subsequent infection.

When *M. pneumoniae* whole organism antigen was used in the CF test, a pre-existing

antibody titer of 1:4 or greater correlated clearly with protection against subsequent *M. pneumoniae* infection ($P < 0.05$, Table 4). As compared with titers less than 1:4, pre-existing antilipid antibody titers of 1:4 or 1:8 were associated with protection against the acquisition of mild *M. pneumoniae* disease ($P < 0.05$, Table 1). These men were treated mainly as outpatients. Some required hospitalization, but they did not have pneumonia. Antilipid antibody titers of 1:16 were clearly related to protection against mild disease as well as severe disease requiring hospitalization ($P < 0.005$,

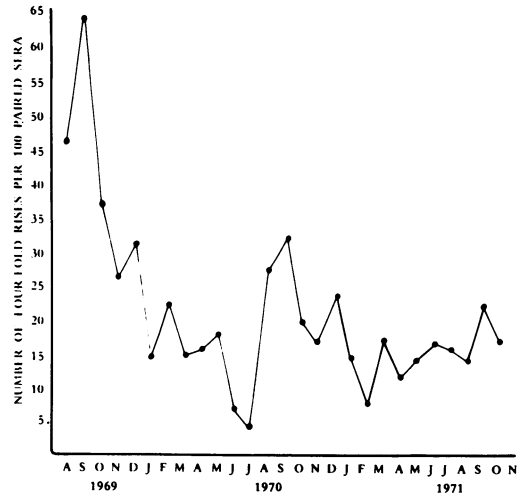


FIG. 2. Relative number of ill trainees with a fourfold rise in whole-organism *M. pneumoniae* complement-fixing antibodies per 100 paired sera. Seroconversions to *M. pneumoniae* paralleled the increase in isolations during the summer months. Seroconversions were detected approximately twice as frequently as isolations of *M. pneumoniae* from patients with acute respiratory disease.

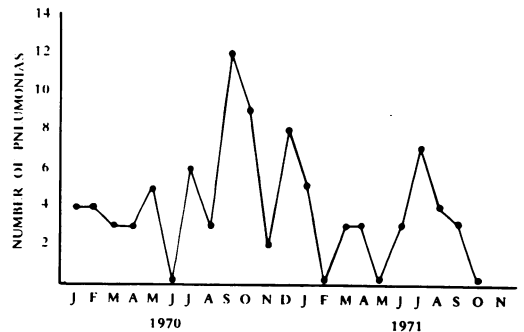


FIG. 3. The number of trainees hospitalized with *M. pneumoniae* pneumonia at the Naval Hospital, Camp Lejeune, also followed a seasonal pattern with peaks in the summer months.

Table 3). However, pre-existing antilipid antibody titers of 1:4 to 1:8 were not associated with protection against pneumonia ($P < 0.70$, Table 1) and accounted for 35% of the patients with pneumonia. In contrast, only 15% of the patients with *M. pneumoniae* disease without pneumonia had intermediate levels of pre-existing antilipid antibody (Table 1).

Relationship of severity of illness to antilipid CF antibody rise. Of interest was the observation that sera obtained from pneumonia patients on arrival at the hospital had higher CF antibody titers than acute-phase sera obtained from trainees ill with *M. pneumoniae* disease but requiring only outpatient care (Table 5). Additionally, the convalescent antibody titer in the trainees with more severe disease was higher than those with milder illness ($P < 0.001$). In *M. pneumoniae* disease, unlike most viral infections, a twofold increase in geometric mean CF antibody titer occurred by the time a patient required hospitalization.

DISCUSSION

This study supports earlier observations of a seasonal factor in both *M. pneumoniae* isolations and disease (5, 25). At Camp Lejeune, *M. pneumoniae* is a more significant cause of pneumonia during the summer months than other agents, such as adenovirus, parainfluenza virus, and influenza strains, detected in military populations. During the summer of 1969, *M. pneumoniae* was isolated from 25% of all trainees reporting with symptoms of acute respiratory disease, and during the summer and fall of 1970, 200 of 296 sera from trainees reporting with acute respiratory disease demonstrated a fourfold rise in *M. pneumoniae* antibody. Although *M. pneumoniae* is isolated year-round at Camp Lejeune, the reasons for its predominance during the summer are little

understood. The high rate of *M. pneumoniae* isolation in the summer months was somewhat exaggerated because adenovirus epidemics at Camp Lejeune increase the total number of trainees cultured in the winter. This phenomenon causes a relative decrease in the rate of recovery of *M. pneumoniae* in the winter as compared with the summer months.

Of trainees studied at Camp Lejeune for acute respiratory disease, 24% of those seroconverting between the acute and convalescent serum specimens had pneumonia. The actual proportion of trainees at Camp Lejeune with *M. pneumoniae* pneumonia was probably higher than 24% because *M. pneumoniae* antibody was frequently high by the time of hospital admission, and a further fourfold rise in antibody titer was not always detected in trainees with pneumonia from whom the organism was recovered. The rate of pneumonia among the trainees who seroconverted was high when compared with rates reported in previous studies (8, 12), and the high rate was probably related to the surveillance techniques used. All hospitalized trainees were studied, whereas studies were carried out on only 5 to 10% of trainees reporting to the dispensary for acute respiratory disease. A more accurate estimate of the pneumonia rate is probably that of Chanock and co-workers, who reported that clinically diagnosed pneumonia occurred in only one of 30 trainees experiencing a seroconversion to *M. pneumoniae* by the fluorescent antibody method (8). Recent studies in the United Kingdom indicated that only 30 to 50% of patients with *M. pneumoniae* "infection" had symptoms, and of these only 10% had pneumonia (12).

The significance of *M. pneumoniae* as a cause of pneumonia and acute respiratory disease in military recruits continues to provide incentive for the development of effective chemotherapeutic or immunological control of the disease. We have shown that, when large groups of trainees are studied, a pre-existing antilipid or anti-whole organism CF antibody titer of 1:4 or greater correlated with protection against naturally acquired infection. Whole organism CF antigen is adequate for serological testing to determine protective antibody titers, although the CF test with lipid antigen would appear to be more sensitive. Live, attenuated or killed *M. pneumoniae* vaccines inducing a CF titer of 1:4 or higher in previously susceptible individuals would, therefore, be expected to protect against natural infection.

It is important to emphasize, however, that although naturally occurring serum CF anti-

TABLE 5. Relationship between severity of illness and *M. pneumoniae* antilipid complement-fixing antibody rise

Serum	Geometric mean titer	
	Trainees treated as outpatients only (37 men)	Trainees requiring hospitalization (45 men)
Preinfection	1.33 ^a	1.82 ^a
Acute	3.09 ^b	3.81 ^b
Convalescent	29.98 ^c	94.70 ^c

^a *t* test, $P > 0.05$.
^b *t* test, $P < 0.05$.
^c *t* test, $P < 0.001$.

body was associated with protection against subsequent illness, it is possible that other factors, such as locally synthesized respiratory tract immunoglobulin A and cell-mediated immunity, may be important in protection against disease. Whether a vaccine given intranasally or parenterally might be capable of inducing such an immune response remains a matter of speculation until further studies are made.

It has been suggested that pre-existing *M. pneumoniae* antibody might play a role in the pathogenesis of pulmonary damage by sensitizing individuals to a more severe subsequent infection (1). However, aside from three trainees with a pre-existing titer of 1:16, who were later hospitalized with *M. pneumoniae* infection, and aside from a larger number of trainees with pre-existing titers of 1:4 to 1:8 who developed pneumonia as compared with trainees who experienced *M. pneumoniae* disease without pneumonia (Table 1, $0.20 > P > 0.10$), we have as yet no statistically significant evidence for a sensitizing effect which correlated with naturally acquired pre-existing antilipid or anti-whole-organism *M. pneumoniae* antibody.

The occurrence and distribution in nature of antigens shared by the membrane of *M. pneumoniae* should be emphasized. Such diverse organisms as *M. pneumoniae* (18), *M. neurolyticum* (14), and *M. mycoides* (4) share antigens which cross-react with antibodies specific for the lipid moiety of the *M. pneumoniae* membrane. Therefore, although antilipid antibodies can be removed by adsorption with *M. pneumoniae* membrane glycolipids, all individuals with pre-existing antilipid antibody need not have had prior *M. pneumoniae* infection. The extent of immunological cross-reaction between antibody to *M. pneumoniae* lipids and other similar antigens in the environment and the extent of human responses to homologous antigens are subjects of current interest and importance in the understanding of the epidemiology of *M. pneumoniae* infections.

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