In Vitro Response of Human Lymphocytes to
Mycoplasma pneumoniae

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In vitro culture and stimulation of human peripheral lymphocytes were employed to investigate the role of cellular immunity in *Mycoplasma pneumoniae* disease. Subjects with documented natural infections served as donors. The lymphocyte response to whole *M. pneumoniae* organisms was determined as incorporation of tritiated thymidine in a semimicro culture system. The range of cellular reactivity stimulated by specific antigen was within the range stimulated by phytohemagglutinin. The difference between responses of subjects with documented infection and serologically negative controls was highly significant. Specific reactivity of peripheral lymphocytes correlated closely with the presence of serum growth-inhibiting antibodies, and both persisted for several years following infection. Serum complement-fixing titers correlated well with lymphocyte stimulability during the first year but antibody, as measured by this technique, tended to disappear in later convalescence. In light of previous studies, which revealed a lack of correlation between humoral antibodies and resistance to reinfection, these results suggest that immunity to *M. pneumoniae* infection is mediated by circulating small lymphocytes.

The role of cellular immunity in *Mycoplasma pneumoniae* disease is unknown although the pathogenesis of natural and experimental infections with this agent has been studied for several years (10). The lack of correlation of the course of infection with the appearance of serum antibodies (12), the enigma of the role of secretory antibodies to *M. pneumoniae* (2, 11, 14), and the relative ineffectiveness of killed vaccines (14) suggest, by the process of exclusion, that cell-mediated immunity could be the most important component of the host immune response to this agent.

Certain observations in previous studies indicate that cellular immunity is stimulated by natural and experimental *M. pneumoniae* infection. The histology of the typical pneumonic lesion, consisting of peribronchial and perivascular lymphocytic infiltrates (9), resembles the lesions of delayed hypersensitivity in other organ systems (32). In the hamster, most of the cells composing the acute lesions of experimental disease contain immunoglobulins, but a definite population of immunoglobulin-negative lymphocytes is also present (15). Furthermore, the pulmonary infiltrates produced by a challenge infection occur in an accelerated sequence (6) and are composed of immunoglobulin-negative small lymphoid cells (15).

Since direct examination of lung pathology is restricted by the rarity of fatalities in this disease (11, 24), in vitro techniques were sought for study of the cell-mediated response. Lymphocyte culture and stimulation with microbial antigens have been investigated in a variety of bacterial (20, 26), fungal (25, 29, 33), viral (27–28), and rickettsial (8) infections and in volunteers infected with *M. pneumoniae* (23). In most of these reports lymphocyte transformation showed a positive correlation with infection and, where tested, with skin tests for delayed hypersensitivity. Although other in vitro tests of lymphocyte function more directly reflect cellular immunity, lymphocyte transformation was more adaptable for clinical studies and is the first step in the assessment of several lymphocyte factors (3).

This report is concerned with the stimulability of lymphocytes from peripheral blood of volunteers in whom natural *M. pneumoniae* infection was documented by microbiological and serological methods. The results provide evidence that circulating small lymphocytes remain reactive to *M. pneumoniae* for at least several years.

MATERIALS AND METHODS

Study population. Volunteers were selected from ongoing studies of acute respiratory disease in this laboratory. Three groups provided subjects: patients admitted to the University of North Carolina Student Infirmary with atypical pneumonia, faculty and

personnel of the Division of Health Affairs, and pediatric patients admitted to N.C. Memorial Hospital. Etiological agents were isolated from throat swabs and identified by methods previously described (7).

Antigens for in vitro lymphocyte stimulation. *M. pneumoniae* strain PI 104166 (12) was grown on a HeLa cell culture in Eagle's minimal essential medium supplemented with 10% horse serum and 2,000 units of penicillin G per ml. The culture was passed three times to eliminate nonreplicating products in the original seed material. The infected tissue cultures were scraped with a rubber spatula into fresh culture medium and frozen at −70 C in 1-ml samples. Uninfected HeLa cultures were similarly processed as antigen controls. Quantitation of thawed samples of the antigen pool yielded 4×10^6 colony-forming units of *M. pneumoniae* per ml. Both antigen and control vials were heated at 56 C for 30 min prior to use. Antigen dilutions, tested in several preliminary lymphocyte culture experiments, revealed no upper limit to the dose-response curve; thus, in all experiments the undiluted antigen was employed. Phytohemagglutinin-P (PHA-P, Difco, Detroit, Mich.), reconstituted from the lyophilized state with tissue culture medium RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), was stored at 4 C and renewed every 4 weeks. From this stock material a fresh 1:10 dilution in RPMI 1640 medium was prepared each week.

Lymphocyte culture and stimulation. Heparinized blood (20 units/ml) was mixed in a disposable plastic syringe 5:1 with Plasmagel (Laboratoire Roger Bellon, Neuilly, France) and allowed to sediment at room temperature. The volume obtained varied from 5 to 30 ml depending on size of the patient and type of experiment. The supernatant plasma and buffy coat were ejected into a sterile, plastic, tissue-culture tube (Falcon Plastics, Los Angeles, Calif.) and centrifuged at $120 \times g$ for 10 min. Supernatant plasma was clarified by higher centrifugation and used for supplementation of culture medium, and a sample was frozen for serological studies. The cell button was resuspended in 10 ml of RPMI 1640 medium and was incubated on a nylon fiber column (semidull type 200, E.I. Dupont de Nemours Co., Wilmington, Del.) for 30 min at 37 C. In pediatric subjects, where lymphocytes comprised greater than 60% of the total leukocytes, this step was bypassed to avoid loss of cells in small samples. The cell population eluted from the column with one volume of RPMI 1640 medium consisted of approximately 85% lymphocytes. The final suspension for a culture was prepared in RPMI 1640 medium supplemented with 15% heat-inactivated autologous plasma. No antibiotics were added. In many experiments a second set of cultures was supplemented with serum from a single donor pool kept frozen at −20 C. [No consistent difference was noticed between cultures with autologous sera and this (MLF) heterologous serum. Results were combined for this study except where one or the other set of cultures was clearly superior in terms of overall stimulation ratios.]

Except where otherwise indicated, 2.5×10^5 lymphocytes were cultivated in 0.2 ml of medium in glass tissue-culture tubes (6 by 50 mm) capped with aluminum foil. Details of the methods employed have been published elsewhere (18). Ten microliters of antigen suspension, or PHA-P, was added to appropriate tubes and incubation was carried out in 4% CO_2 for 5 days at 37 C with a relative humidity of 85%. 1.0 μCi of thymidine-*methyl-³H* (³H-Tdr) with a specific activity of 1.9 Ci/mmole (Schwarz/Mann, Orangeburg, N.Y.) was added to each tube for the final 4 hr of incubation. After centrifugation at $800 \times g$ for 10 min at 4 C, the supernatant medium was aspirated and the cell button was processed for determination of retained radioactivity in a liquid scintillation spectrometer (18).

Serological methods. Complement-fixing (CF) antibodies in twofold dilutions of plasma were determined by a previously described micromethod (17) using the chloroform-methanol-extracted lipid antigen from *M. pneumoniae* (22). Comparison of titers obtained with paired samples of serum and heparinized plasma showed no significant difference when specimens were handled as described above. (The addition of fresh heparin just before testing for CF antibodies did reduce the titers approximately fourfold; however, storage of plasma samples at 4 C or −20 C inactivated heparin as evidenced by formation of a fibrin clot upon thawing.) Growth-inhibiting (GI) antibodies were determined in a micromethod based on pH changes (phenol red) accompanying growth of serially diluted organisms during a 1-week period of incubation. Units of growth inhibition represent the number of twofold organism dilutions showing no color change in the presence of a constant amount of serum as compared to serum-free controls (16).

Computation of data. Multiple cultures were prepared from each donor sample to allow three or four replicate tests for each antigen and its paired control. Tubes which occasionally failed to show ³H-Tdr incorporation were disregarded. The arithmetic mean counts per minute of each set of determinations were converted to log_{10} for computation of group means and normal distribution since the cell populations tended to behave in a geometric fashion (18). The ratio of stimulated to unstimulated paired cultures (log-stimulated counts per minute—log control counts per minute) was determined within each experiment to eliminate errors introduced by day-to-day variations in the level of ³H-Tdr incorporation.

RESULTS

Response of human peripheral lymphocytes to PHA and M. pneumoniae antigen. Varying numbers of lymphocytes were cultured in 0.2-ml volumes with a constant dose of PHA (18) to determine the optimum cell concentration for this system. Results of several experiments with 1.0, 2.5, and 5.0×10^5 cells per tube are shown in Table 1. A 5-day incubation was chosen to allow maximum stimulation of blast transformation in the presence of microbial antigens. According to these data, there was a wide range of

TABLE 1. *Response of human peripheral lymphocytes to phytohemagglutinin-P in vitro*

Lymphocytes cultured[a] (× 10⁵)	No. of donors tested	³H-Tdr uptake (log₁₀ counts/min[b])				Stimulation index (log PHA-log control)	
		Cell contro		PHA stimulated[c]			
		GM	SD	GM	SD	GM	SD
1.0	6	2.06 (114)	±0.32	4.52 (32,900)	±0.07	2.46 (288)	±0.34
2.5	36	2.820 (662)	±0.281	4.246 (17,620)	±0.280	1.426 (26.7)	±0.353
5.0	17	2.994 (986)	±0.392	4.181 (15,160)	±0.286	1.186 (15.3)	±0.447

[a] Indicated number of nylon column-separated lymphocytes in 0.2 ml of RPMI 1640 medium supplemented with 15% autologous serum; 5-day incubation with 4-hr terminal exposure to 1.0 μCi of ³H-Tdr.

[b] Arithmetic mean of triplicate determinations for control and stimulated cultures used for calculation of geometric mean (GM) and standard deviation (SD); antilog shown in parentheses is mean counts per minute and ³H-Tdr is thymidine-*methyl*-³H.

[c] Ten microliters of 1:10 dilution of stock phytohemagglutinin-P (PHA-P) (Difco).

TABLE 2. *Response of human peripheral lymphocytes to Mycoplasma pneumoniae in vitro*[a]

No. of lymphocyte donors	No. of tests	³H-Tdr uptake (log₁₀ counts/min)[b]				Stimulation index (log agn-log control)	
		Hela cell control[c]		*M. pneumoniae* antigen			
		GM	SD	GM	SD	GM	SD
Serologically negative subjects (7)	7	2.897 (790)	±0.332	3.079 (1,200)	±0.458	0.182 (1.5)	±0.152
Serologically positive subjects with documented infections (11)	16	3.095 (1,240)	±0.395	4.030 (10,700)	±0.363	0.993 (8.6)	±0.27

[a] 2.5 × 10⁵ nylon column-separated lymphocytes cultured for 5 days in 0.2 ml of RPMI 1640 medium with 15% human serum; 4-hr terminal exposure to 1 μCi of ³H-Tdr.

[b] See footnote b, Table 1.

[c] Ten microliters of *M. pneumoniae*-infected HeLa cell suspension containing 10⁶ colony-forming units/ml. Control tubes received 10 μliters of uninfected cell suspension.

cell concentration for expression of PHA stimulation in this system.

In several of the experiments in Table 1, lymphocytes were stimulated with *Candida albicans*, streptokinase-streptodornase, and tuberculin PPD antigens (*not shown*). Donors with positive cutaneous-delayed hypersensitivity to these antigens were found to have lymphocyte responses in the lower PHA range at cell concentrations of 5.0 and 2.5 × 10⁵/0.2 ml but not in tubes containing only 10⁵ lymphocytes. Therefore, a 2.5 × 10⁵ concentration of lymphocytes was selected as the standard for further studies to preserve economy of cells without forfeiting antigen responsiveness.

Table 2 illustrates results of lymphocyte stimulation with *M. pneumoniae* antigen in donors known to be serologically negative or in whom infection and an antibody response had been documented. In the latter group, repeated bleedings were obtained in three individuals and account for the 16 sets of data on 11 subjects. The difference between stimulated and unstimulated cultures in the control groups was negligible, whereas the difference for previously infected subjects was greater than eightfold. The mean stimulation indexes for the two groups differed by 0.751 log₁₀ or a ratio of 5.6.

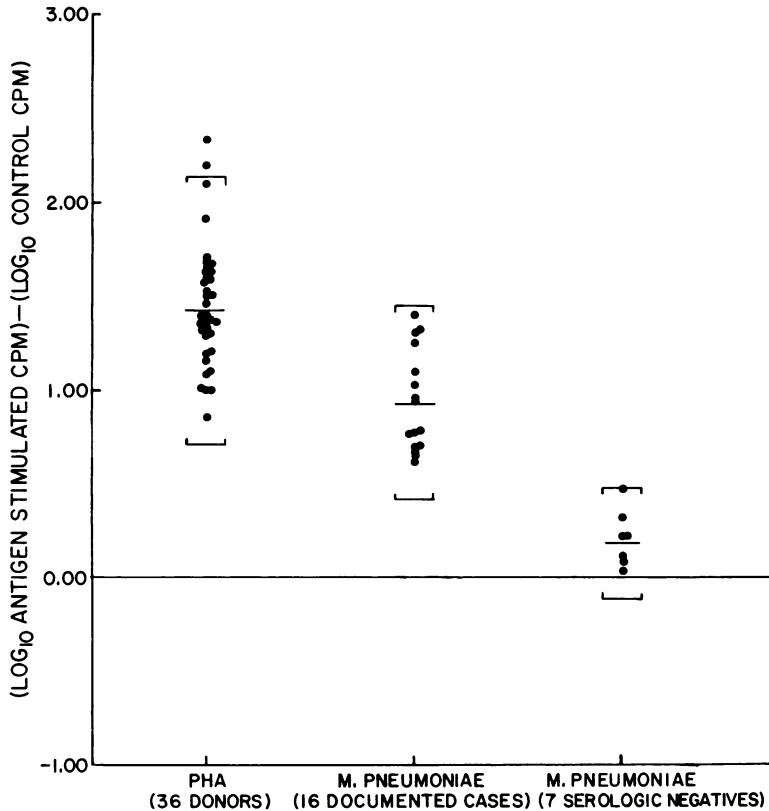The data in Tables 1 and 2 are presented graphically in Fig. 1. Based on the mean PHA response,

FIG. 1. *Thymidine-methyl-^{3}H incorporation of human lymphocytes stimulated with phytohemagglutinin-P and Mycoplasma pneumoniae. Mean logarithm of stimulation indexes $\pm$ 2 standard deviations indicated by brackets.*

± 2 standard deviations, a fivefold or greater stimulation index occurred with over 95% frequency. Thirteen of the *M. pneumoniae*-immune cases responded to homotypic antigen within the PHA stimulation range. All of the 16 serologically positive cases showed a higher response than the serologically negative controls ($P < 0.001$, rank sum method). Thus, it was concluded that a threefold (0.5 $\log_{10}$) or greater stimulation index for *M. pneumoniae* antigen was unlikely to occur in cultures of nonimmune cells and, therefore, this level of responsiveness was significant in this system.

Correlation of M. pneumoniae serum antibody levels with peripheral lymphocyte culture response. In 34 separate peripheral lymphocyte culture (PLC) experiments, CF and GI antibodies were determined on the supernatant plasma. Subjects for these studies included those presented in the foregoing paragraphs and other volunteers whose history of exposure to *M. pneumoniae* had not been documented. Figure 2 illustrates the relationship of the GI antibody level and PLC response in each of the subjects
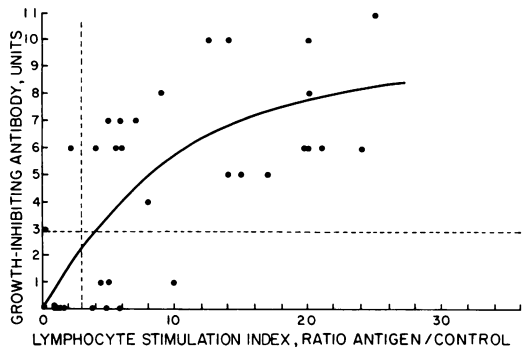

FIG. 2. *Correlation between serum growth inhibition and in vitro lymphocyte response to Mycoplasma pneumoniae. Dotted line indicates lower limits of significance for the coordinates. The curve was drawn on a series of points determined by calculating the means of grouped coordinates.*

tested. If 3 units is taken as the significant level for GI antibody (16, 17), only two individuals with elevated antibodies failed to show significant lymphocyte stimulation. Of the 12 subjects with
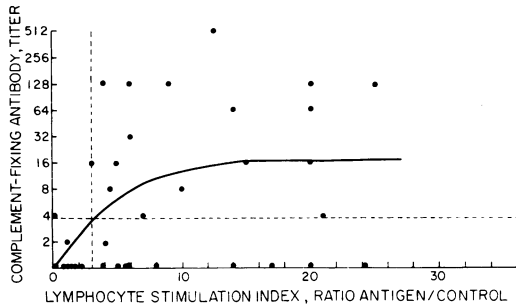
FIG. 3. *Correlation between serum complement-fixing antibody and in vitro lymphocyte response to Mycoplasma pneumoniae antigen. See legend Fig. 2.*

0 or 1 unit of antibody, six had a threefold or greater stimulation index. These data revealed a high degree of correlation between the presence of serum antibody and the PLC response. However, the in vitro PLC method appeared to be more sensitive for detection of reactivity to *M. pneumoniae* than measurement of serum growth-inhibition capacity. As mentioned below, the tendency for specific lymphocyte reactivity to persist for several years also supports this observation.

Figure 3 presents the relationship of CF antibody levels and lymphocyte responsiveness to *M. pneumoniae* in the same subjects. The large number of serological negatives responding in PLC and the scatter of all points indicate a poor correlation for this pair of tests compared with the previous figure. However, if time were added as a third dimension, nearly all CF-negative sera with significant PLC reactivity would be found in late convalescence. All but one person with a CF titer of 1:4 or greater responded in PLC; most, with 1:4 or greater titers, were studied in the first year of convalescence.

Duration of PLC response to M. pneumoniae.

The temporal relationships of antibody and cellular immune responses following infection are shown in Table 3. Serologically negative subjects are presented in lieu of acutely ill patients. This was necessitated by the difficulty of identifying cases prior to development of pneumonia and the 7-day period required for cultivation of the etiological agent. Six young adults with proven infection and pneumonia were studied within 4 weeks of their first symptoms. All six had elevated CF and GI antibodies and five yielded a stimulation index greater than 3.0. Four of these subjects were restudied after 2 months and three, again, after 6 months. As indicated in the table, the level of antibody and PLC reactivity was generally sustained throughout this period of convalescence. Further follow-up was precluded by the academic calendar and accounts for the absence of observations beyond 6 months.

To determine the duration of these parameters of immunity following *M. pneumoniae* pneumonia, subjects studied prior to development of the PLC method were contacted. Six persons, in whom infection had been documented 4 to 7 years previously, were found to have low or undetectable levels of one or both antibodies. Their PLC response, however, was in the same range as subjects studied during the first year after infection occurred. Nine volunteers, in whom the acute infection was undocumented, were presumed to be immune on the basis of elevation of at least one type of antibody. The lymphocyte response to *M. pneumoniae* antigen in these individuals also was in the range found during convalescence from documented disease.

DISCUSSION

In this study, *M. pneumoniae* antigen stimulated tritiated-thymidine incorporation in cul-

TABLE 3. *Duration of immune reactivity to Mycoplasma pneumoniae after natural infection*

Time since infection was documented	No. of subjects tested	Serum antibody levels				Peripheral lymphocyte stimulation index	
		CF titer[a]		GI units[b]			
		−log₂ Mean	Range	Mean	Range	Mean	Range
No infection	7	0.57	(2–4)	0	(0)	1.4	(0.8–2.9)
1 Month	6	6.5	(8–256)	7.6	(4–10)	9.10	(2.5–23)
2 Months	4	5.5	(16–128)	8.3	(7–10)	9.4	(2.5–20)
6 Months	3	5.3	(2–256)	9.0	(6–11)	13.5	(10–20)
4–7 Years	6	1.4	(2–32)	2.9	(0–7)	7.2	(3.9–21)
Undocumented illness	9	1.9	(2–32)	4.1	(0–7)	8.3	(2.4–16)

[a] Complement fixation, reciprocal end point dilution.
[b] Growth inhibition capacity; see text for derivation.

tures of peripheral lymphocytes from volunteers with a documented history of infection. Such cellular reactivity was absent in serologically negative subjects but was present in nearly all persons with specific antibodies. The appearance of maximum lymphocyte reactivity to the etiological agent within the first few months of convalescence and the persistence of responsiveness over the ensuing years paralleled closely the changes seen in GI antibody levels in these and previously reported investigations (17). These observations suggest that a population of antigen-sensitive lymphocytes remains in circulation for a prolonged period following naturally acquired *M. pneumoniae* disease.

Lymphocyte transformation in response to *M. pneumoniae* has been demonstrated previously in human volunteers experiencing an experimental infection (23). Only subjects with tetrazolium reduction-inhibition (TRI) antibody titers of 1:16 or greater showed a significantly increased blast transformation in the presence of *M. pneumoniae* antigen, suggesting that the TRI test (analogous to the GI test) was more sensitive to previous experience with the organism. Since the period of observation in that experiment was only 26 days, the duration of lymphocyte reactivity was not determined.

Although the above experiments indicate that a portion of the circulating pool of lymphocytes becomes reactive to the infecting organism after *M. pneumoniae* pneumonia, the role of such cells in clearing the infection and in maintaining a state of subsequent immunity is unknown. Early during the acute illness it is likely that a portion of the cells within the pulmonary infiltrates is capable of antibody formation (15). However, since thymic-dependent small lymphocytes (T cells) comprise the majority of circulating lymphoid cells, blood samples obtained at a time remote from the acute infection should contain very few antigen-sensitive cells capable of antibody production (31). In this context, the lymphocyte culture system employed in this study served as an index of cell-mediated immunity.

Although the PLC response to *M. pneumoniae* may represent T cell-mediated immunity, the application of more explicit methods is required to prove this concept. Several techniques are available for demonstrating the biological activity of specific products secreted by lymphocytes (3). None of these has been successfully applied to this disease model, but this would appear to be a most promising area for future research on the nature of immune response to *M. pneumoniae*.

The data presented in this report relate to several areas of present interest, one of which concerns the frequency of reinfection with *M. pneumoniae*. If repeated infections were responsible for the maintenance of immune reactivity, one would expect antibody levels to remain moderately elevated. Although Steinberg et al. found serological evidence of reinfection in a study of Marine recruits (30), the gradual decline of titers in the present study suggests that this occurs infrequently. Further evidence of the rarity of reinfection is the paucity of documented cases (19).

It has been proposed that *M. pneumoniae* disease is actually a secondary immune reaction, a preceding asymptomatic and, therefore, undetectable infection being required to sensitize the host (4). This concept was based on the finding of antibodies to *M. pneumoniae* by the highly sensitive complement-mediated killing test and a radioimmunoprecipitation method in subjects who lack evidence of specific immunity by conventional methods. The immunoprecipitin technique employed by Brunner et al. was based upon the complexing of labeled organisms with antibodies in human serum. Although there are several precipitable antigens in *M. pneumoniae* (21), none of which have been related to biological activity in vivo, the significance of immunoprecipitin titers in human serum is unclear. Likewise, the complement-mediated killing test is of questionable biological significance, for antibodies can be found with this test in normal animals (21). Thus, the increased sensitivity of these two methods may have been gained at the expense of specificity. Our data do not support the concept of *M. pneumoniae* disease as a hypersensitivity reaction. The course of experimental *M. pneumoniae* infection (9, 12), the correlation of the PLC response with natural infection, and its persistence for at least several years suggest that most persons experiencing *M. pneumoniae* disease never before have been infected.

A subject of considerable interest at the present time is the development of *M. pneumoniae* vaccines (5). As in other superficial infections of the respiratory tract, immunity induced by natural disease or attenuated live vaccine is superior to that resulting from injection of killed organisms (14). Although the highly specific GI antibodies do not correlate directly with this state of immune resistance, it is possible that the presence of circulating sensitized lymphocytes would. Critical experiments on the role of this parameter of cellular immunity will depend upon the application of the PLC technique in vaccine trials with both live and killed organisms.

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