Isolation and Characterization of Fractions of *Mycoplasma pneumoniae*

II. Antigenicity and Immunogenicity

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ABSTRACT

SOBESLAVSKY, O. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), B. PRESCOTT, W. D. JAMES, AND R. M. CHANOCK. Isolation and characterization of fractions of *Mycoplasma pneumoniae*. II. Antigenicity and immunogenicity. J. Bacteriol. 91:2126-2138. 1966.—Chemical and chromatographic fractions of disrupted *Mycoplasma pneumoniae* organisms were examined for serological and immunogenic activity. Complement-fixing activity was associated with lipid components, whereas precipitin activity was chiefly associated with polysaccharide components. When chemically extracted lipids were separated by thin-layer silica gel chromatography, only three of the nine fractions exhibited complement-fixing activity. Although lipids were highly active serologically, they were only weakly immunogenic. However, lipids combined with protein in lipoprotein complexes were highly immunogenic, stimulating high levels of complement-fixing, indirect-hemagglutinating, and growth-inhibiting antibodies. The specificity of these antibodies was directed chiefly against the serologically active lipid constituents of the organism. It was suggested that these serologically active lipids are present at the sites on the limiting membrane of *M. pneumoniae* at which antibody acts to inhibit growth of the organism. Only protein fractions adsorbed to tanned erythrocytes. The main function of protein in the indirect-hemagglutination reaction appeared to be that of serving as a carrier for the serologically active lipids.

In the accompanying report, we described the separation of *Mycoplasma pneumoniae* into a number of chemical and chromatographic fractions (10). Chemical extraction of the organism yielded one lipid fraction, three protein fractions, and four polysaccharide fractions. The major protein fraction was found to contain a small quantity of lipid. Four separate fractions were obtained when the supernatant fluid from a sonically disrupted suspension of organisms was chromatographed on a Sephadex G-25 column. These fractions contained mostly protein and some polysaccharides, and the one fraction tested for lipid was found to contain 15% of such material.

This portion of the study was initiated to determine the nature of the various antigens of *M.*

pneumoniae which react in the different serological procedures used for assay of antibody. In addition, we were interested in defining the nature of the antigens of this organism which stimulate antibodies measurable by the different serological techniques. Such information would have relevance to our basic understanding of this important respiratory-tract pathogen, and to the future preparation of purified vaccines containing only those antigens which stimulate protective antibodies.

MATERIALS AND METHODS

M. pneumoniae strain. The FH strain of *M. pneumoniae* was isolated in embryonated eggs by Liu from a patient with atypical pneumonia (9). The organism was subsequently passaged 305 times in artificial cell-free media in this laboratory (2). Harvests from the 295th passage through the 305th passage were used for this study.

Cultivation medium for preparation of crude starting

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material and methods of fractionation. The methods used for cultivation of *M. pneumoniae* were described in the preceding paper (10). The procedures used for chemical fractionation and Sephadex chromatography were also described in the preceding paper (10).

Complement-fixation test. Lyophilized *M. pneumoniae* fractions obtained by means of chemical or chromatographic fractionation were prepared as 1% solutions or suspensions in phosphate-buffered saline (PBS) and then tested for complement-fixing activity. Lipid fractions were dissolved in a small amount of propylene glycol before these materials were diluted to 1% in PBS. All fractions were tested by the micro complement-fixation technique (11). Two exact units of complement were used. The antigen-serum-complement mixtures were incubated overnight at 4 C prior to addition of sensitized erythrocytes.

The preparation of standard *M. pneumoniae* antigen was described previously (3). Briefly, organisms (FH strain) were grown aerobically in broth medium at 34 C for 16 days, and then 0.5% phenol was added and the suspension was reincubated for an additional 4 days. The suspension was then heated at 56 C for 30 min, and this material constituted the complement-fixing antigen.

Indirect-hemagglutination test. The *M. pneumoniae* fractions tested by complement fixation were also tested for their capacity to sensitize tanned sheep red cells (6, 12). For purposes of comparison, a sonically treated suspension of whole organisms was also tested for indirect-hemagglutination activity.

The method of sensitization of tanned sheep red blood cells and of antigen titration, and the technique of the indirect hemagglutination test were described in detail previously (12). Briefly, sheep erythrocytes were washed three times in PBS. The cells were "tanned" by incubating a 4% suspension with an equal quantity of 1:20,000 tannic acid in PBS for 10 min and were washed three times in PBS by further centrifugation. The antigen was diluted in twofold steps (starting at 1 in 8) in PBS, and samples of the dilutions were mixed with equal quantities of the 2% washed tanned cells. The cell-antigen mixtures were incubated for 20 min at 37 C and then centrifuged at 1,500 rev/min for 10 min. The cell deposits were resuspended in PBS to which heat-inactivated (56 C, 30 min) normal rabbit serum had been added to give a 1:200 final concentration of serum. After recentrifugation the cells were resuspended in the 1:200 normal rabbit serum to produce a 1% suspension of sensitized tanned sheep erythrocytes. For controls, tanned sheep red cells were carried through the same procedures, except that they were treated with PBS instead of antigen. Antisera were heated at 56 C for 30 min and diluted in 1:200 normal rabbit serum. Tests were carried out with 0.025-ml volumes in disposable plastic microtitration plates (Cooke Engineering Co., Alexandria, Va.). The final reaction mixtures were incubated at 34 C for 2 hr; 75% agglutination of sensitized red cells was considered the end point for determination of both antigen and serum titer.

Test of inhibition of indirect hemagglutination. The test was devised to quantitate the capacity of various

fractions of *M. pneumoniae* to combine with the antibodies which agglutinate sensitized red cells.

Serial twofold dilutions of each fraction were made in test tubes and to each dilution was added an equal volume of *M. pneumoniae* rabbit antiserum diluted to contain 8 units of indirect hemagglutinating antibody. The diluent was normal rabbit serum diluted 1:200 in PBS. The same serum dilution mixed with the diluent was used for a control. The mixtures were then incubated, with occasional shaking, for 1 hr at 37 C. Then each of the mixtures was transferred in 0.025-ml amounts into microtiter disposable plates (with "V"-shaped cups) by use of a microtiter dropper. A volume of 0.025 ml of tanned sheep red blood cells sensitized with a sonically treated suspension of whole organisms was then added to each cup. The plates were then sealed with Scotch cellophane tape and incubated for 2 hr at 34 C.

The highest dilution of a fraction which inhibited 75 to 100% of the agglutinating activity produced by 8 units of antibody was considered as 1 unit of inhibitory activity.

Growth-inhibition test (tetrazolium reduction inhibition test). The test was a modification of that described by Jensen (Bacteriol. Proc., p. 70-71, 1964). It was performed in disposable microtiter plates with "U"-shaped cups. Heat-inactivated (56 C, 30 min) serum was diluted in twofold steps in 0.025-ml quantities by use of microtiter loops. The diluent consisted of the broth medium used for cultivation of *M. pneumoniae*, except that glucose and phenol red were omitted, and 2,3,5-triphenyltetrazolium chloride was added to a final concentration of 0.05%. An inoculum of 0.025 ml of *M. pneumoniae* (FH strain, passage 227) was then added with a microtiter dropper to each cup, followed by a final 0.15 ml of the medium. The plates were then sealed with Scotch cellophane tape, wrapped in tin foil to prevent light penetration, and incubated at 34 C. The inoculum was a stock frozen (-70 C) suspension of *M. pneumoniae* diluted in broth medium to a concentration of approximately 10^4 colony-forming units (CFU) per 0.025 ml. The suspension of organisms was titrated at the time of each test, and a standard *M. pneumoniae* human convalescent serum was included in each test as a positive serum control. The results of the test were recorded when the dilution of the organisms used in the test reduced tetrazolium, and when an indicated dilution of the standard serum inhibited growth of the organisms. This occurred with the above-mentioned inoculum after 5 to 6 days of incubation.

Test of inhibition of growth inhibition. Unlike the growth-inhibition test, which measures the capacity of homologous antibody to inhibit the growth of the organism, this test was devised to measure the capacity of fractions to block the inhibitory activity of *M. pneumoniae* antibody by combining with such antibody.

Serial twofold dilutions of various fractions were made in broth diluent in test tubes, and to each dilution was added an equal volume of *M. pneumoniae* rabbit antiserum diluted to contain 8 units of growth-inhibitory antibody. Serum dilutions mixed with broth diluent served as controls. The mixtures were

then incubated, with occasional shaking, for 1 hr at 37 C. Then 0.025-ml quantities of each of the fraction-serum mixtures were transferred into microtiter disposable plates ("U"-shaped cups) by use of a micro-dropper. The cups were then inoculated with 0.025 ml of a suspension containing approximately 10^4 CFU of *M. pneumoniae* (FH strain, passage 227). Finally, 0.15 ml of broth medium was added to each cup; the plates were then sealed with Scotch cellophane tape and wrapped in tin foil to prevent light penetration. Incubation was at 34 C. A titration of the inoculum was performed at the time of each test.

Results were recorded when the dilution of organisms used in the test reduced tetrazolium, and when 8 units of antibody inhibited this reduction. The highest dilution of a fraction capable of blocking the growth-inhibitory activity of 8 units of antibody was considered as 1 unit of inhibitory activity.

Gel-diffusion test. All of the chemically extracted and chromatographic fractions were tested for precipitin activity by the agar gel-diffusion technique. Each of the fractions was tested as a 10% suspension or solution. The tests were performed by use of agar gel immuno-plates purchased from Hyland Laboratories, Los Angeles, Calif. Reservoir center-to-center distance was 4 mm. After reagents had been added to the cups, the plates were incubated at 34 C in a humidified chamber. The lines were observed under oblique light. Drawings of the precipitation lines were made as soon as they were first observed, and later as the lines continued to develop.

Immune sera. A convalescent serum from a volunteer infected with *M. pneumoniae* was used to measure the complement-fixing activity of the various chemical and chromatographic fractions. The serum was collected 3 weeks after infection with the PI 898 strain of *M. pneumoniae*. This strain was recovered and propagated only in monkey kidney tissue culture (4). Serum taken from the volunteer prior to infection did not contain complement-fixing ($<1:10$) or growth-inhibiting ($<1:1$) antibodies, whereas the convalescent phase serum contained high levels of complement-fixing (1:320), indirect hemagglutination (1:2,048), and growth-inhibiting (1:2,048) antibodies. Furthermore (at a dilution of 1:20), the convalescent serum did not contain complement-fixing antibodies for the other mycoplasma species that infect man (*M. hominis* type 1, *M. salivarium*, *M. fermentans*, *M. orale* type 1, and *M. orale* type 2).

Two rabbit hyperimmune antisera were used in this study. The first antiserum was used to measure the complement-fixing and indirect-hemagglutination activity of the various fractions. It was also used in the antibody-blocking tests. This antiserum was prepared by repeated injection of rabbits with an *M. pneumoniae*-infected chick embryo lung suspension. This serum did not contain antibodies for the medium constituents present in the broth medium used for the cultivation of *M. pneumoniae*, but did contain high levels of *M. pneumoniae* antibodies measurable by complement fixation (1:1,024), indirect hemagglutination (1:2,048 to 1:4,096), and growth inhibition (1:20,480). The serum exhibited a heterotypic complement-fixation reaction with only one of the

other human mycoplasma species (*M. salivarium*), and in this instance the heterotypic serum titer (1:64) was considerably lower than the homotypic titer (1:1,024). The maximal heterotypic indirect hemagglutination reactions exhibited by the serum were low (1:20) when compared with the homologous titer (1:2,048 to 1:4,096). Indirect hemagglutination antigens were prepared from sonically disrupted organisms; thus, internal as well as external components were available as antigens in this test.

The second rabbit antiserum was used for the agar gel-diffusion studies. Rabbits were immunized with a suspension of *M. pneumoniae* grown in rabbit infusion broth supplemented with rabbit serum. The antiserum produced three precipitin lines in agar gel when tested with a concentrated suspension (800 times) of disrupted *M. pneumoniae*. The serum did not produce precipitin lines when tested with concentrated suspensions (800 times) of disrupted heterotypic organisms which were reactive (three or more precipitin lines) with their homologous antisera.

In the complement-fixation, indirect-hemagglutination, and growth-inhibition tests, the *M. pneumoniae* immune sera were used at a dilution which was eight-fold greater than the serum end point. At these dilutions, the antisera did not exhibit heterotypic reactivity.

Immunization of animals with *M. pneumoniae* fractions. Antisera to the fractions of *M. pneumoniae* were produced in rabbits and guinea pigs. For immunization, 1% suspensions or solutions of the lyophilized fractions in PBS were used.

Rabbits were injected intradermally at multiple sites with a mixture of 1 ml of fraction and 1 ml of complete Freund's adjuvant. The same volume of injected material, but mixed with incomplete adjuvant, was given intradermally 3 weeks later. After an additional 3-week interval, 1 ml of fraction was injected intraperitoneally, and the animals were bled out 1 week later.

The method for guinea pig immunization was essentially the same, except that the material injected consisted of 0.5 ml of *M. pneumoniae* fractions and 0.5 ml of adjuvant. For purposes of comparison, guinea pigs were also immunized with 1 and 10% suspensions of *M. pneumoniae* disrupted by sonic treatment.

RESULTS

Antigenic activity of fractions. The various fractions extracted from *M. pneumoniae* were tested for their activity in the complement-fixation and indirect-hemagglutination reactions. In addition, they were tested for their capacity to block the effect of indirect-hemagglutinating and growth-inhibiting antibodies.

Chemical fractions. After sonic treatment of *M. pneumoniae* at 10 kc for 1 hr, the suspension was centrifuged at $63,000 \times g$ for 1 hr. The resulting sediment was then treated as indicated in the preceding paper, and a series of fractions were obtained. With one exception (polysaccharide frac-

tion 1), complement-fixing activity was demonstrated only in those fractions which contained lipid (Table 1). Not all lipid fractions, however, exhibited complement-fixing activity. Six of the nine fractions obtained after silica gel thin-layer

chromatography of the chemically extracted lipid fraction lacked complement-fixing activity. Similarly, three of the five fractions obtained after thin-layer chromatography of the lipids extracted from the Sephadex G-25 fraction 1 lacked com-

TABLE 1. *Serological reactivity of chemically extracted fractions*

Material	Units of activity per mg of dry wt ^a			Units of inhibitory activity per mg of dry wt ^d	
	CF ^b		IHA		
	Conva- lescent human serum ^c	Hyper- immune rabbit serum ^c	Hyper- immune rabbit serum ^c	IHA	Growth- inhibition
<i>Lipid</i>					
(a) Lipid fraction (chemical extract).....	2,048	2,048	0	256	256
(b) Thin layer chromatographic fractions of lipid frac- tion (a)					
Fraction 1.....	1,280	NT ^e	NT	640	160
Fraction 2.....	2,560	NT	NT	640	320
Fraction 3.....	640	NT	NT	320	160
Fractions 4-9.....	0	NT	NT	0	0
(c) SG 25 fine, chromatographic fractions of lipid frac- tion					
Fraction 1.....	4,096	4,096	NT	256	256
Fraction 2.....	0	0	NT	0	0
(d) Lipid extracted from protein 2.....	640	NT	0	320	640
(e) Lipid extracted from sephadex G-25 fraction 1.....	10,240	NT	0	2,560	2,560
(f) Thin-layer chromatographic fractions of lipid ex- tracted from Sephadex G-25 fraction 1					
Fraction 1 ^f	10,240	NT	NT	640	1,280
Fraction 2 ^g	5,120	NT	NT	640	640
Fractions 3-5.....	0	NT	NT	0	0
<i>Polysaccharide</i>					
Polysaccharide fraction 1.....	0	64	0	0	0
Polysaccharide fraction 2.....	0	0	0	0	0
Polysaccharide fraction 3.....	0	0	0	0	0
Polysaccharide fraction 4.....	0	0	0	0	0
<i>Protein</i>					
(a) Protein fraction 1.....	0	0	0	0	0
(b) Protein fraction 2.....	64	32	256	128	32
(c) Protein fraction 2 chromatographed on Sephadex G-25; fraction 1.....	256	128	64	128	32
(d) Protein 2 with lipid removed.....	0	NT	32	64	0
(e) Protein 3.....	0	0	64	128	16
Starting material, sonically treated suspension of <i>Myco- plasma pneumoniae</i>	1,965	984	123	492	984

^a CF = complement fixation; IHA = indirect hemagglutination.

^b Antigenic activity measured with 8 units of convalescent human serum or hyperimmune rabbit serum.

^c None of the antigens reacted with acute phase serum or preimmunization serum.

^d One unit of inhibitory activity = highest dilution of fraction capable of inhibiting 8 units of indirect-hemagglutinating or growth-inhibiting antibody in rabbit hyperimmune serum.

^e Not tested.

^f This fraction corresponds to the fraction 1 of lipid b.

^g This fraction corresponds to the fraction 3 of lipid b.

plement-fixing activity. Initially, the protein 2 fraction exhibited CF activity, but the activity was lost after removal of lipid. On a weight basis, the reactive lipid fractions were more active than protein fraction 2, which contained a small quantity of lipid (2%).

Only protein fractions exhibited indirect-hemagglutinating activity. It was not possible to sensitize tanned red cells with the various lipid or polysaccharide fractions. However, lipid fractions which exhibited complement-fixing activity also reacted with the antibodies which agglutinate sensitized red cells, i.e., they blocked indirect hemagglutination. These findings suggest that certain of the lipid fractions contain one or more of the antigenic determinants involved in the indirect hemagglutination reaction; however, these determinants are not able to adsorb to tanned red cells. Consistent with this view was the decrease in indirect hemagglutinating activity of protein fraction 2 after removal of lipid.

With the possible exception of protein fraction 3, which was not tested for lipid, only those chemical fractions which contained lipid and exhibited complement-fixing activity blocked the growth-inhibitory activity of specific rabbit antiserum. Removal of lipid from protein fraction 2 resulted in a loss of reactivity of this material with growth-inhibitory antibodies. On a weight basis, the reactive lipid fractions were more active than protein fraction 2, which contained only 2% lipid by weight.

Chromatographic fractions. The supernatant fluid obtained from the sonically treated suspension of *M. pneumoniae* was subjected to chromatographic separation. The first two fractions obtained after chromatography on Sephadex G-25, as well as the rechromatographed fraction 1 (Sephadex G-100), exhibited a high level of CF activity (Table 2). This activity, at least for fraction 1, could be ascribed to the lipid component (15% of the total weight) of the fraction. When lipid was removed from fraction 1, the material lost its complement-fixing activity. Fractions 3 and 4 exhibited low levels of complement-fixing activity, which could be detected only when the fractions were tested with human convalescent serum.

The first two fractions were capable of sensitizing tanned red cells, whereas fractions 3 and 4 were not. The erythrocyte-sensitizing activity of fraction 1 was not completely dependent upon the lipid component, but the fraction was less active when lipid was removed. In addition, the concentration of antibody required to agglutinate red cells sensitized with the lipid-free fraction 1 was 16 times greater than that needed by the original Sephadex G-25 fraction 1.

Fractions 1 and 2 and the rechromatographed fraction 1 (Sephadex G-100) were active in blocking the indirect-hemagglutination reaction. Probably lipid was involved in this activity, since fraction 1 with lipid removed was considerably less active than the original fraction. Fractions

TABLE 2. Serological reactivity of chromatographic fractions

Material ^a	Units of activity per mg of dry wt ^b			Units of inhibitory activity per mg of dry weight ^d	
	CF		IHA	IHA	Growth inhibition
	Conva-lescent human serum ^c	Hyper-immune rabbit serum ^c	Hyper-immune rabbit serum ^c		
(a) SG25 fraction 1	4,096	2,048	512	1,024	1,024
(b) SG25 fraction 1 rechromatographed SG100	8,192	2,048	256	2,048	2,048
(c) SG25 fraction 1 with lipid removed	0	NT ^e	32	64	0
(d) SG25 fraction 2	2,048	512	512	1,024	1,024
(e) SG25 fraction 3	32	0	0	32	32
(f) SG25 fraction 4	64	0	0	0	0
Starting material, sonically treated suspension of <i>Mycoplasma pneumoniae</i> (calculated for dry wt.)	1,965	984	123	492	984

^a SG25 and SG100 = Sephadex G-25 and G-100.

^b Antigen activity measured with 8 units of convalescent human serum or hyperimmune rabbit serum. CF = complement fixation; IHA = indirect hemagglutination.

^c None of the antigens reacted with acute-phase serum or preimmunization serum.

^d One unit of inhibitory activity = highest dilution of fraction capable of inhibiting 8 units of indirect-hemagglutinating or growth-inhibiting antibody in rabbit hyperimmune serum.

^e Not tested.

3 and 4 exhibited little or no antibody-combining activity.

Fractions 1 and 2 were active in combining with growth-inhibiting antibody, whereas fractions 3 and 4 were not. Lipid appeared to be responsible for the antibody-blocking activity of fraction 1, since removal of lipid from this fraction led to a complete loss of reactivity with growth-inhibitory antibodies.

On a dry-weight basis, the complement-fixation indirect-hemagglutination, and antibody-combining activities of the Sephadex G-25 fraction 1 and its rechromatographed fraction were similar to those of the starting material. This suggests that the active components of the organism were not selectively concentrated by chromatography on the Sephadex G-25 or G-100 column.

Recovery of serological activity in the various fractions. After chemical or chromatographic fractionation of a sonically treated suspension of *M. pneumoniae*, almost all of the complement-fixing activity was recovered in four fractions—the lipid fraction and protein fraction 2 derived from the sediment of the *M. pneumoniae* suspension, and the Sephadex G-25 fractions 1 and 2 derived from the supernatant fluid of the suspension. Similarly, almost all of the growth-inhibitory antibody-combining activity was recovered in these four fractions. Approximately 45% of the CF activity of the starting material was recovered in these fractions (Table 3). Recovery of growth-inhibitory antibody-combining activity in these fractions was somewhat less (25%). It should be emphasized that these values are only approximate, since the error of the titration procedures is at least two-fold. Despite this source of error, it would appear that considerably more complement-fixing and growth-inhibitory antibody-blocking activities were recovered in the chromatographic fractions (derived from the supernatant

fluid of the sonically treated *M. pneumoniae* suspension) than in the chemical fractions (derived from the sediment of the sonically treated suspension).

Comparison of several fractions by complement fixation. In the first paper of this series, it was shown that the lipids extracted from the sediment of the sonically treated organisms, from Sephadex G-25 fraction 1, and from protein fraction 2 exhibited some chromatographic similarities. After silica gel thin-layer chromatography, each of these extracts was found to separate into five to nine discrete fractions. The lipid extract of the sediment of the sonically treated organisms yielded nine fractions, but only the first three exhibited complement-fixing activity (Table 1). When the first of the fractions was rechromatographed on silica gel, four separate fractions were obtained. Rechromatography of the first fraction from the lipid of Sephadex G-25 fraction 1 yielded only three fractions. However, these fractions appeared to be identical to three of the fractions obtained after rechromatography of the first fraction of the lipid from the sediment of the sonically treated organisms.

The relationships of the lipid fractions were examined further by complement fixation. The lipids and the materials from which they were extracted were cross-tested by use of antisera prepared against the lipid fraction from the sediment of the sonically treated organisms, Sephadex G-25 fraction 1, and protein fraction 2.

The lipid extract from the sediment of the sonically treated organisms and that obtained from the Sephadex G-25 fraction 1 appeared to be closely related antigenically (Table 4). The former lipids were distinct serologically from the lipids of protein fraction 2. The Sephadex G-25 fraction 1 lipids exhibited a one-way relationship with the lipids of protein fraction 2; antiserum to the former reacted with the latter antigen, but not

TABLE 3. *Recovery of serological activity in chemical extracts and chromatographic fractions*

Material	Dry weight	Total no. of units of activity in indicated material ($\times 10^6$)	
		CF	Inhibition of growth inhibition
Starting material (suspension of sonically treated organisms).....	g 10.24	20	10.1
Chemical fractions of sediment of sonically treated organisms			
Lipid fraction.....	1.015	2.1	0.3
Protein fraction 2.....	2.145	0.1	0.1
Chromatographic fractions of supernatant fluid of sonically treated organisms			
Sephadex G-25 fraction 1.....	1.075	4.4	1.1
Sephadex G-25 fraction 2.....	1.12	2.3	1.1
Total recovered in the above 4 fractions	5.335	8.9	2.6

TABLE 4. Comparison of serological reactivity of the lipid and protein fractions extracted from Sephadex G-25 fraction 1 and protein fraction 2

Material	Reciprocal of complement-fixing antibody titer of the antiserum prepared against		
	Lipid extracted from <i>Mycoplasma pneumoniae</i> *	Sephadex G-25† fraction 1	Protein fraction 2†
Lipid extracted from <i>M. pneumoniae</i>	16	64	>4
Sephadex G-25 fraction 1	4	256	32
Lipid separated from Sephadex G-25 fraction 1	32	64	>4
Protein separated from Sephadex G-25 fraction 1	>1	32	4
Protein fraction 2	4	16	16
Lipid separated from protein fraction 2	>1	64	128
Protein separated from protein fraction 2	>1	16	16

* Rabbit antiserum.

† Guinea pig antiserum.

vice versa. These findings suggest that there may be quantitative differences in the distribution of the serologically active lipids in the Sephadex G-25 and protein 2 materials. Possibly, qualitative differences also exist.

Comparison of precipitating antigens in various fractions. Eight of the chemically extracted fractions and five of the chromatographic fractions were tested by the agar gel-diffusion technique for the presence of precipitating antigens. In addition, a 20% (wet weight) suspension of sonically treated organisms was tested for comparison. Hyperimmune rabbit antiserum was placed in the center cup, and the material to be tested for precipitin activity was placed in one of five peripheral cups which surrounded the center cup. One fraction was compared with another by placing the test materials in adjacent peripheral cups.

The suspension of whole organisms produced three precipitation lines in agar gel. For convenience, we have designated these lines A, B, and C. Three of the five chromatographic fractions tested produced precipitation lines. Sephadex G-25 fraction 1 and this fraction rechromatographed on Sephadex G-100 each produced three precipitation lines. Sephadex G-25 fraction 2 produced two lines, whereas fractions 3 and 4 did not produce a reaction in agar gel. The three lines of both Sephadex G-25 fraction 1 and its rechromatographed fraction gave reactions of identity with lines A, B, and C produced by the sonically treated suspension of whole organisms. The two lines produced by Sephadex G-25 fraction 2 exhibited reactions of identity with lines A and B.

Three of the chemically extracted fractions produced a precipitin reaction in agar gel. The lipid extract (from the sediment of the sonically treated organisms) produced one precipitation line which was transient. This line developed approximately

5 hr after the lipid fraction was added to the agar; however, the line was not detectable 24 hr later. Protein fraction 2 produced one line which persisted. Polysaccharide fraction 2 produced two precipitation lines, both of which persisted. The other five chemical fractions tested—polysaccharide fractions 1, 3, and 4, and protein fractions 1 and 3—did not produce precipitation lines.

The line produced by protein fraction 2 gave a reaction of identity with line A of the sonically treated organism suspension. The lines of polysaccharide fraction 2 were related to, but not identical with, lines B and C of the sonically treated organism suspension. When polysaccharide fraction 2 and the sonically treated organism suspension were placed in adjacent cups, the two lines produced by the former material were not continuous with lines B and C; instead, spurring was observed.

Because of its transient nature, the line produced by the lipid extract (from the sediment of the sonically treated organisms) was difficult to compare with the precipitation lines produced by the other fractions. In one test, the lipid precipitin was shown to be distinct from the two polysaccharide precipitins. The relationship of the lipid precipitin to the other precipitating antigens was not tested successfully.

Immunogenic activity. Eight chemically extracted fractions and four chromatographic fractions were tested for their capacity to stimulate the production of complement-fixing indirect-hemagglutinating, and growth-inhibiting antibodies in animals. In addition, protein fraction 2 chromatographed on Sephadex G-25 and the rechromatographed Sephadex G-25 fraction 1 (Sephadex G-100) were studied. All of the fractions were injected into guinea pigs, whereas only five fractions were tested in rabbits.

TABLE 5. Immunogenic activity of chemically extracted and chromatographic fractions

Material (1% suspension)	Reciprocal of antibody titer of sera from animals immunized with fractions ^a							
	Rabbit				Guinea pig			
	Complement fixation		IHA ^d	Growth inhibition	Complement fixation		IHA	Growth inhibition
	Homologous antigen ^b	Standard antigen ^c			Homologous antigen	Standard antigen		
<i>Chemically extracted fractions</i>								
Lipid	32 0	16 0	64 4	0 ^e 0	0 ^f 0	0 ^f 0	64 0 ^f	4 4
Polysaccharide 1					512 >1,024	512 256	256 16	2 0 ^e
Polysaccharide 2					16 0	0 0	64 16	2 2
Polysaccharide 3					0 0	0 0	4 0	2 2
Polysaccharide 4					0 0	32 0	0 0	4 2
Protein 1					128 32	64 32	2,048 512	2 2
Protein 2	16 16	128 64	>4,096 >4,096	0 0	16 8	32 16	>4,096 >4,096	2 2
Protein 2, chromatographed on Sephadex G-25 fraction 1					>1,024 >1,024	16 32	>4,096 >4,096	2 2
Protein 3					0 0	0 0	2,048 512	2 0
<i>Chromatographic fractions</i> Sephadex G-25 fraction 1	>1,024	256	>4,096	128	512 256	16 8	>4,096 >4,096	64 32
<i>Chromatographic fractions</i> re-chromatographed on SG-100, fraction 1	1,024 512	256 128	>4,096 >4,096	128 32	256 128	16 0	>4,096 >4,096	64 4
Sephadex G-25 fraction 2	>1,024 >1,024	>1,024 256	>4,096 >4,096	128 64	>1,024 >1,024	512 64	>4,096 >4,096	4 2
Sephadex G-25 fraction 3					>1,024 1,024	256 128	>4,096 >4,096	2 2
Sephadex G-25 fraction 4					>1,024 1,024	0 8	2,048 256	8 0
Starting material (sonically treated suspension of <i>Mycoplasma pneumoniae</i> 10%.....)						1,024 1,024	>4,096 >4,096	32 32
1%						64 64	>4,096	4 2

^a Each value represents the antibody titer of a single animal.^b Antigen used for immunization.^c Whole suspension of *M. pneumoniae*, phenol-treated.^d Indirect hemagglutination.^e 0 = <1:4.^f 0 = <1:2.

High levels of complement-fixing antibodies, i.e., 1:256 to 1:1,024 or greater, were stimulated by polysaccharide fraction 1, protein fraction 2 chromatographed on Sephadex G-25, Sephadex G-25 fraction 1 and its rechromatographed fraction (Sephadex G-100), and Sephadex G-25 fractions 2, 3, and 4 (Table 5). In several instances, a high titer of complement-fixing antibody developed for the homologous (i.e., the immunizing) antigen but not for the standard antigen; this phenomenon was observed with the chromatographed protein fraction 2, the Sephadex G-25 fraction 1, and its rechromatographed fraction, and Sephadex G-25 fraction 4. These differences may reflect a difference in the quantitative or spatial distribution of active components in the immunizing material and in the standard complement-fixing antigen.

Moderate levels of complement-fixing antibody, i.e., 1:32 to 1:128, were stimulated by protein fractions 1 and 2. It is of interest that the lipid fraction, which was the most active material in the complement-fixation reaction, stimulated complement-fixing antibody in only one of six injected animals. One of two rabbits developed a moderate level of complement-fixing antibody, whereas two guinea pigs shown in Table 6 and two others failed to develop such antibody.

High levels of indirect hemagglutinating antibodies were stimulated by those fractions which contained high concentrations of protein, i.e., protein fractions 1, 2, and 3, and Sephadex G-25 fractions 1, 2, 3, and 4. Lipid fraction and polysaccharide fractions 1 and 2 stimulated low levels of such antibody. Polysaccharide fractions 3 and 4 were essentially nonimmunogenic with respect to indirect-hemagglutination antibody.

Significant levels of growth-inhibitory antibody were stimulated by only three of the fractions studied—Sephadex G-25 fraction 1 and its rechromatographed fraction (Sephadex G-100), and Sephadex G-25 fraction 2. The last of these fractions stimulated moderate levels of growth-inhibitory antibodies in rabbits, but not in guinea pigs, whereas the other two fractions were immunogenic in both species. The other chemically extracted or chromatographic fractions all stimulated the development of low levels of growth-inhibitory activity in guinea pigs; sera collected before immunization did not exhibit such inhibitory activity. The specificity of these low-level growth-inhibitory responses remains to be established, especially in view of the diverse nature of the fractions which stimulated such responses.

DISCUSSION

In this study, a number of chemical and chromatographic fractions of *M. pneumoniae*

were investigated for their antigenicity and serological reactivity. An interpretive summary of the results obtained is given in Table 6. Using these data, we will attempt to describe the nature of the components responsible for various antigenic and immunogenic activities of the organism. It should be emphasized that the chemical fractionation procedure offered a number of opportunities for antigens to be denatured and for antigens to be artificially partitioned between fractions.

Complement-fixing activity of *M. pneumoniae* were associated predominantly with its lipid components. These findings are consistent with those of Kenny, who extracted complement-fixing antigen from *M. pneumoniae* with several lipid solvents (8). In addition, we found that the first polysaccharide fraction, which was lipid-free, exhibited a low level of complement-fixing activity when tested with hyperimmune rabbit serum. However, this fraction did not react with convalescent human serum. Several of the protein-containing fractions (protein fraction 2 and Sephadex G-25 fraction 1) exhibited complement-fixing activity, but this activity was produced by the lipid components of these fractions. When lipids were removed from these fractions, complement-fixing activity was lost.

When examined by thin-layer chromatography, the lipid extract of the sediment of the sonically treated organisms separated into nine discrete fractions. Only the first three of the fractions exhibited complement-fixing activity. It is of interest that these three fractions did not contain cholesterol, which is a major constituent of sterol-requiring mycoplasmas. Preliminary analysis indicates that the three active fractions contain phospholipids. The first of the active thin-layer chromatographic fractions was not homogeneous, since rechromatography on silica gel yielded four discrete fractions.

After thin-layer chromatography, the lipids from protein fraction 2 and Sephadex G-25 fraction 1 each yielded fractions which corresponded to the serologically active first and third fractions obtained from the lipid extract derived from the sediment of the sonically treated organisms. This resemblance was more than chromatographic, since the fractions separated from the lipids of Sephadex G-25 fraction 1 which corresponded to the serologically active fractions of the lipids from the sonically treated organisms also exhibited complement-fixing activity.

Chemically extracted lipids did not adsorb to tanned sheep erythrocytes. However, lipid appeared to be involved in the indirect hemagglutination reaction, since the lipids active in complement fixation also combined with and blocked the action of the antibodies which agglutinate

sensitized red cells. Although protein by itself was capable of adsorbing to tanned erythrocytes, it was less important than lipid in the reaction with agglutinating antibodies. Thus, the reactive lipid fractions were more active in inhibiting indirect hemagglutination, on a weight basis, than was protein free from lipid. Also, protein fraction 2 and Sephadex G-25 fraction 1 lost considerable indirect-hemagglutination activity when lipids were removed from these fractions. Taken together, these findings suggest that the sensitizing moiety in the indirect hemagglutination reaction is a lipoprotein; the protein component is probably responsible for adsorption to tanned red cells, whereas the lipid component is mainly responsible for the reaction with specific agglutinating antibodies. Tully previously described sensitization of erythrocytes with protein extracts from a number of different mycoplasmas (13).

The lipid components which are active in complement fixation and which react with indirect-hemagglutinating antibody also appear to be present at the sites at which antibodies produce a growth-inhibitory effect. Thus, polysaccharide fractions and protein fractions free from lipid did not react with growth-inhibitory antibodies, whereas fractions containing serologically active lipid components blocked the growth-inhibitory effect of antiserum.

The chemically extracted (purified) lipid fraction which exhibited marked *in vitro* serological activity was weakly immunogenic after injection of rabbits and guinea pigs. Low-level stimulation of complement-fixing and indirect-hemagglutinating antibodies was observed, but growth-inhibitory antibody did not develop. The poor immunogenicity of these lipids is not surprising, since lipids in general are known to be ineffective stimulators of antibody (7). In contrast to purified lipids, lipids bound to proteins in lipoprotein complexes (Sephadex G-25 fraction 1 and protein fraction 2) were found to be moderately or highly immunogenic. Moderate levels of growth-inhibitory antibodies and high levels of complement-fixing and indirect-hemagglutinating antibodies were stimulated by the lipoprotein-containing Sephadex G-25 fraction 1. It should be emphasized that the specificity of the growth-inhibitory antibodies was directed against the lipid components of the lipoprotein complex. It is probable that growth-inhibitory antibodies act upon the limiting membrane of the organism. For this reason, it is likely that the lipoproteins which react with growth-inhibitory antibodies occupy a peripheral position on the organism—most likely on the triple-layered unit membrane which surrounds the organism (5).

Polysaccharide fractions were not active in

complement fixation except for fraction 1, which exhibited low-level activity. The polysaccharide fractions did not participate to a significant extent in the indirect-hemagglutination reaction. Furthermore, the fractions did not react with growth-inhibitory antibody. The major activity of the polysaccharides was exhibited in the precipitation reaction. Similar findings have been reported for polysaccharides extracted from *M. mycoides* (14). In the present experiments, polysaccharide fraction 2 produced two precipitation lines when tested by the agar gel-diffusion technique. Chemically extracted lipid (from the sediment of the sonically treated organisms) and protein fraction 2 each produced only one precipitation line. The lipid precipitin was completely distinct from the two polysaccharide precipitins. A sonically treated suspension of the whole organism produced three precipitation lines in agar gel, and two of these lines appeared to be related to the precipitation lines produced by polysaccharide fraction 2.

Protein fraction 2 initially exhibited complement-fixing activity, but this was lost when lipid was removed from the fraction. Protein, free from lipid, could sensitize tanned sheep red cells, but for maximal indirect-hemagglutinating activity a lipoprotein complex was required. The main function of protein in the indirect-hemagglutination reaction appears to be that of serving as a carrier for the active lipid components. Protein probably serves a similar function in contributing to the immunogenicity of the active lipids.

The first two fractions recovered from the Sephadex G-25 column exhibited a high level of reactivity in the complement-fixation and indirect-hemagglutination reactions. These fractions also combined effectively with growth-inhibitory antibodies, and were the only fractions which stimulated moderate levels of such antibodies in guinea pigs and rabbits. In each of these serological and immunogenic activities, the first two chromatographic fractions were considerably more potent than the chemically extracted protein fraction, which contained a small quantity of lipid (2%).

The finding that the first and second chromatographic fractions stimulated the development of growth-inhibitory antibodies is significant. Previously, we have shown that growth-inhibitory antibodies correlate well with resistance to *M. pneumoniae* illness (1). Possibly, chromatographic techniques similar to those used in this study may prove useful in the development of a purified, inactivated *M. pneumoniae* vaccine for the prevention of cold agglutinin-positive atypical pneumoniae.

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