A Radioimmunoprecipitation Test for Detection of *Mycoplasma pneumoniae* Antibody (37261)

HELMUT BRUNNER[1] AND ROBERT M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014; the Research Foundation of Children's Hospital of the District of Columbia, Washington, D. C. 20009; the World Health Organization International Reference Center for Human Mycoplasmas

More sensitive serologic techniques are needed for the assay of antibody to *Mycoplasma pneumoniae* in order to unravel certain unusual aspects of the behavior of this organism in man. Recently, a mycoplasmacidal test (MCT) for detection of antibody to *M. pneumoniae* was modified by us (1, 2). This method proved to be considerably more sensitive than complement fixation and metabolism inhibition for measurement of *M. pneumoniae* antibody while at the same time retaining specificity. Because the mycoplasmacidal assay determines complement dependent antibody the measurement of immunoglobulins of the IgA class—the major immunoglobulin in local body secretions—is not possible. Also, like some virus neutralization procedures, it is a rather laborious test. Therefore, we developed a radioimmunoprecipitation test (RIP) which employs microtiter equipment and requires small amounts of reagents. This method is based on the double antibody technique, and depends upon the observation that the antigenic properties of gamma globulins are retained when they complex with an antigen. It is possible to separate bound from unbound antigen by precipitating the antigen–antibody complexes with antisera to gamma globulins. This method has several advantages over precipitation with ammonium sulfate: ammonium sulfate precipitates *M. pneumoniae* antigen, whereas the double antibody method does not affect the antigen in this manner. Further the double antibody method can be easily adapted to the assay of large numbers of samples and it permits the identification of the immunoglobulins involved through use of specific antisera for the separate immunoglobulin classes (3).

Materials and Methods. Organisms. Strain PI 1428 of *Mycoplasma pneumoniae* and strain DC-63 of *M. hominis* were employed in their 5th and 6th laboratory passages, respectively (2). *M. neurolyticum* type A and *M. fermentans* strain PG 18 were used for experiments concerned with the specificity of the RIP reaction. *M. neurolyticum* type A was kindly supplied by Dr. Joseph G. Tully, Laboratory of Microbiology, NIH.

Growth medium and culture conditions. The organisms were grown in mycoplasma broth medium containing 20% agamma horse serum (Baltimore Biologic Laboratories, Cockeysville, MD) instead of normal horse serum (4, 5). This medium was supplemented for *M. pneumoniae* with 1% glucose, 0.002% phenol red, 0.05% thallium acetate and 1000 units of penicillin G/ml. For the growth of *M. hominis* 1% l-arginine–HCl was added instead of glucose. The pH of the medium for *M. pneumoniae* was adjusted to 7.6 with 0.1 *N* sodium hydroxide; the medium for *M. hominis* was adjusted to pH 6.6 with 0.1 *N* hydrochloric acid. *M. pneumoniae* was grown at 37° on the glass surface of 5 liter Povitsky bottles containing 1 liter of medium (6). *M. hominis* was grown at 37° in screw capped 32 oz bottles containing 500 ml of medium. The growth medium for *M. neurolyticum* and *M. fermentans* contained 1% bovine PPLO serum fraction (Difco Laboratories, Detroit, MI) instead of

[1] Supported in part by a fellowship from the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany. Present address: Institute for Medical Microbiology, 63 Giessen, Schubertstr. 1, West-Germany.

agamma horse serum and no thallium acetate. Growth conditions were the same as described for *M. pneumoniae* but incubation at 37° was for only 3 days (7).

Antigen preparation. For the labeling of *M. pneumoniae* 0.1 μCi/ml of oleic-acid-1-^{14}C (sp act 62 mCi/mmole) and 0.1 μCi/ml of palmitic acid-1-^{14}C (sp act 57.7 mCi/mmole) were added to the growth medium 24 hr after the inoculation of the organisms and incubation at 37° was continued until the medium started to change color, usually after 5–7 days (8, 9). The radioactive compounds were obtained from Amersham/Searle, Arlington Heights, IL.

M. hominis was added at the same time as the labeled compounds. Although the major serologic antigen of *M. hominis* is not a lipid, organisms labeled with ^{14}C-fatty acids did bind antibody and such antigen–antibody complexes could be precipitated by antiserum to human globulins.

^{14}C-Labeled *M. pneumoniae* was harvested by scraping the cell sheet adhering to the glass surface of each bottle into 30 ml of Veronal-buffered saline after decanting the medium and washing the cell sheet 5 times with 100 ml amounts of buffer. *M. hominis* was harvested after 36–48 hr of incubation by centrifugation of the medium at 16,000*g* for 2 hr at 4° in a Sorvall RC-2 centrifuge. The pellet was washed twice in buffer by centrifugation at 36,000*g* for 15 min at 4°. The antigens were distributed in small quantities in glass vials and stored at −70° until used. The specific activity of the antigens was 380,000 dpm/mg total protein for *M. pneumoniae* and 68,200 dpm/mg total protein for *M. hominis*. The method of Lowry *et al.* (10) was used for determination of protein. A single lot of each antigen was used for all experiments.

Human sera. Sera from volunteers collected prior to and 28 days after infection with the PI 898 strain of *M. pneumoniae* and similar sera from volunteers infected with *M. hominis* strain DC-63 were employed to standardize the test procedures (11, 12). All sera were heated at 56° for 30 min before use.

Preparation of purified human gamma globulin. To 20 ml of pooled human sera 10 ml of saturated ammonium sulfate was added dropwise with constant stirring. After 30 min at room temperature the precipitate was sedimented and subsequently washed twice with 50 ml amounts of 40% ammonium sulfate. The precipitate was then resuspended in distilled water and dialyzed overnight against 0.1 *M* Veronal-buffered saline at pH 7.4. The dialyzed material was further purified by agar-block electrophoresis as described by Olsen, McCammon and Yohn (13). The proteins were localized by removing a longitudinal section of the agar block which was fixed with a mixture of methanol, water and acetic acid (4.5:4.5:1; v/v). The gamma globulin containing part of the agar was cut out and the proteins were extracted by at least 10 freeze–thaw cycles. The IgA, IgG, and IgM content of gamma globulin preparation was determined by radial-immunodiffusion (14). The gamma globulin preparation had 1.9 mg/ml total protein and contained 0.53 mg/ml IgA, 0.90 mg/ml IgG and 0.15 mg/ml IgM. The preparation was filtered twice through 800 nm pore size Millipore filters, for the purpose of sterilization.

Immunization of guinea pigs. Germ-free guinea pigs (Hartley strain) 8 wk of age were injected intradermally at several sites with 0.5 ml purified gamma globulin suspended in an equal amount of complete Freund's adjuvant (H37Ra, Difco Laboratories, Detroit, MI). Three weeks later the animals received 0.5 ml of purified gamma globulin intradermally suspended in an equal volume of incomplete Freund's adjuvant. One week later 0.5 ml of purified gamma globulin was administered intraperitoneally. After 1 wk the animals were test-bled and the concentration of antibody to human IgA, IgM and IgG determined by double immunodiffusion using purified human immunoglobulins (Meloy Laboratories, Springfield, VA) as antigens (15). When visible precipitation lines with all three gamma globulin classes were observed, the immunization process was terminated. Usually a second intraperitoneal injection of antigen was necessary to elicit a satisfactory antibody response. One week after the last administration of antigen the animals were sacrificed, the serum separated, pooled, distributed in small quantities and stored at −20° until used.

Radioimmunoprecipitation test (RIP). The radioimmunoprecipitation test (RIP) was performed by the microtiter method as modified by Sever (16), using U-bottom plastic plates (Cooke Engineering, Inc., Alexandria, VA). A preliminary description of the method was reported previously (17). The radiolabeled antigen and sera were diluted in Veronal-buffered saline (pH 7.4) containing 0.15 m*M* Ca^{2+}, 0.5 m*M* Mg^{2+} and 1% bovine serum albumin (VBS-BSA). Fourfold serum dilutions were prepared and each serum was tested in duplicate. Twenty-five microliters of a filtered (450 nm pore size Millipore) suspension of the ^{14}C-labeled antigen, (approx 600 to 1000 dpm) was added to 25 μl of diluted serum. The plates were placed in a humidified sealed box and incubated for 60 min at 37° and 24 hr at 4°. Then 150 μl of a 1:5 dilution of anti-human gamma globulin (produced in germ-free guinea pigs) were added. In some experiments burro anti-human whole serum purchased from Meloy Laboratories (Springfield, VA) was used. The optimal dilution of the antiserum was determined beforehand in a checkerboard test with a human serum known to contain antibody to *M. pneumoniae*. After addition of the antiserum the plates were again incubated for 60 min at 37° and then overnight at 4°. The plates were then centrifuged at 1000 rpm for 10 min at 4° in an International clinical centrifuge. One hundred microliters of the supernatant were withdrawn with an Eppendorf pipette and placed into glass scintillation vials. After addition of 0.5 ml of NCS solubilizer (Amersham/Searle, Des Plaines, IL) the closed vials remained at room temperature until the fluid was dissolved, usually after approximately 1 hr. Toluene scintillator (10 ml) containing PPO and POPO (Liquifluor, New England Nuclear, Boston, MA) was added. This produced a clear fluid and no significant quenching was observed as determined by internal standards. The vials were then placed into a liquid scintillation counter (LS250, Beckman Instruments, Inc., Fullerton, CA). The counting efficiency for ^{14}C was 90%. After dark and temperature adaptation for several hours each vial was counted until a preset error of 2% was reached but not longer than 20 min if the radioactivity was very low. Background counts were automatically subtracted by the liquid scintillation counter. Ten buffer controls and two antiserum controls were included in each test. The standard deviation of the controls did not exceed 16 dpm. The radioactivity deposited in the precipitate was determined by subtracting the cpm made by 100 μl of supernatant from a well containing the test serum from the value observed for 100 μl of control supernatant. The percentage binding of antigen to antibody was calculated by the formula: $[(S_c - S_t)/S_c] \times 100 = X[\%]$, where S_t was the radioactivity (cpm) in the supernatant from a test serum containing well and S_c the mean (cpm) of control supernatant fluids. The titer of the test serum was calculated as the highest serum dilution which produced 33% binding. A positive control serum was included in each test. If the titer of this serum showed a more than twofold discordance from the anticipated value the test was considered invalid.

Complement fixation (CF), metabolism inhibition (MI), immunofluorescence (IMF). Complement fixation and metabolism inhibition tests and indirect immunofluorescence were performed as described previously (11, 18–20). For the CF test 1.8 units of complement were used, with overnight fixation at 4°.

Mycoplasmacidal test (MCT). The test for determination of mycoplasmacidal antibody to *M. pneumoniae* was performed as described previously (2).

Extraction of lipids. Lipids of *M. pneumoniae, M. neurolyticum* and *M. fermentans* were extracted as described by Kenny and Grayston (21). The lipids were dissolved in absolute ethanol and diluted in VBS-BSA to yield a final concentration of 500 μg/ml.

Blocking experiments. Checkerboard titrations of a human convalescent serum (25 μl) and either purified membrane glycolipids of *M. pneumoniae* or lipids of *M. pneumoniae, M. neurolyticum* or *M. fermentans* (25 μl) were performed in microtiter plates. The plates were incubated for 1 hr at 37° and overnight at 4°. ^{14}C-Labeled *M. pneumoniae* antigen (25 μl) was then added and the procedure continued as described for the RIP test. Purified membrane glycolipids of *M.*

pneumoniae were prepared as described previously and kindly supplied by Dr. Benjamin Prescott, Laboratory of Microbiology, NIH (22, 23).

Results. 1. Requirement for a homogeneous monodispersed suspension for RIP. As indicated in Table I filtration of labeled *M. pneumoniae* through a 450 nm pore size Millipore filter was necessary to obtain a homogeneous suspension of organisms which did not sediment during the incubation period of the procedure and the subsequent centrifugation at 1000 rpm. For *M. hominis* filtration through a 800 nm pore size filter was sufficient. Following filtration of either mycoplasma approximately 80 to 90% of the radioactivity and a comparable number of viable organisms were retained on the filter. The filtered antigens remained dispersed during the test procedure unless antigen–antibody complexes were formed and precipitated by an antiserum to human gamma globulin.

2. Influence of antibody concentration on amount of labeled M. pneumoniae *precipitated.* Figure 1 shows that more than 80% of the labeled antigen was still precipitated at a dilution of 1:1024 by a convalescent human *M. pneumoniae* antiserum. The degree of binding decreased thereafter reaching 33% at a 1:65,536 serum dilution. The smaller amount of antigen bound at high antiserum concentrations is probably due to the prozone phenomenon, well known in precipitation reactions.

3. Effect of cations in buffer. Neither the use of various buffers nor the addition of divalent cations (Ca^{2+}, Mg^{2+}) altered the RIP antibody titers of human antiserum. These results suggest that complement or complement components were not necessary for the binding of antibody to antigen in the RIP test.

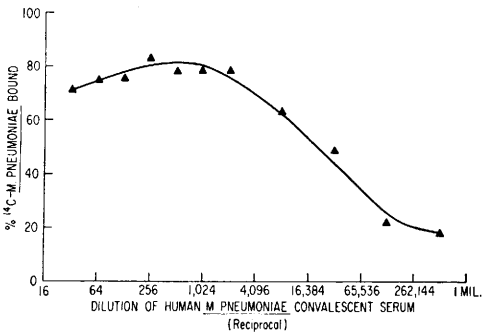

FIG. 1. Binding of ^{14}C-labeled *M. pneumoniae* antigen by various concentrations of a human convalescent serum. Anti-human whole serum produced in a burro was used to precipitate the complexes.

4. Aquisition of RIP antibody to M. pneumoniae *by age.* As expected, RIP antibody to *M. pneumoniae* was detected with low frequency in infants 7–12 mo of age (Fig. 2). At this time maternal antibody should have disappeared from the infant's serum. An increase in the proportion of positive sera and mean titer of antibody was observed for children 13–36 mo of age. The level of RIP antibody in sera from adults was higher than that found for young children.

TABLE I. Effect of Filtration of ^{14}C-Labeled Mycoplasma on Sedimentability of Radiolabel at 1000 rpm During RIP Procedure.

Filtration of antigen	Radioactivity (%) which sedimented at 1000 rpm (10 min) after 48 hr incubation of antigen and buffer at 4°	
	M. pneumoniae	*M. hominis*
Not filtered	60–70	80
Filter (nm), 800	30	0
650	8	ND[a]
450	0	ND[a]

[a] Not done.

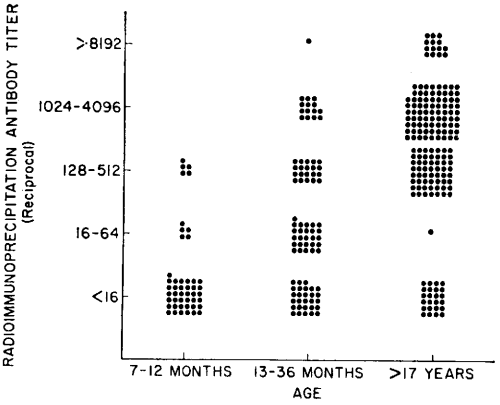

FIG. 2. Aquisition of RIP antibody to *M. pneumoniae* by age.

5. Sensitivity. The RIP test was used to study sera from 10 human volunteers who were infected via the nasopharynx with *M. pneumoniae* strain PI 898 (11). The resulting antibody titers are indicated in Table II and compared to the results of conventional methods of antibody assay and the mycoplasmacidal test. The titers obtained in the RIP test were usually considerably higher than those obtained by CF, MI or IMF. Most RIP titers were also higher than the corresponding MCT antibody titer. Like the MCT procedure the RIP method revealed antibody in preinfection sera which was not detected by the conventional methods. Except for no. 9, each of the volunteers who exhibited an increase in antibody titer by the conventional methods also showed an increase in RIP antibody titer.

The fold rises in titers of *M. pneumoniae* antibody measured by different techniques were compared using the Spear rank correlation. Very good correlation was seen between RIP and MCT. The RIP also correlated well with MI and IMF. However, the correlation of RIP and glycolipid CF antibodies was less impressive suggesting that antigens other than glycolipids were also measured by the radioimmunoprecipitation technique.

6. Reproducibility. When sera of 4 volunteers were tested for RIP-antibody in three separate tests on different days the variation of *M. pneumoniae* antibody titer was never more than twofold (Table III).

7. Specificity. Sera of two volunteers infected with *M. pneumoniae* and two volunteers infected with *M. hominis* were compared for their RIP antibody titers. As shown in Table IV a significant rise was observed only with the homologous antigen.

TABLE II. Antibody Responses of Volunteers Infected with *M. pneumoniae* as Measured by Complement Fixation (CF), Metabolism Inhibition (MI), Immunofluorescence (IMF), Mycoplasmacidal Test (MCT), and Radioimmunoprecepitation Test (RIP).

	Reciprocal of preinfection (above) and postinfection (below) antibody titer as measured by					
Volunteer	CF (whole cell antigen)	CF (glycolipid antigen)	MI	IMF	MCT	RIP
1	<2	4	<2	10	25	32
	64	128	96	160	20,830	4096
2	<2	<4	<2	<10	27	16
	32	128	12	80	3200	512
3	<2	<4	3	10	63	32
	128	256	48	>160	1728	2048
4	<2	<4	<2	<10	28	256
	64	32	48	160	1297	8192
5	<2	<4	<2	<10	4	8
	<2	<4	24	160	233	2048
6	<2	<4	3	10	82	64
	16	16	24	40	359	512
7	<2	<4	12	10	53	64
	<2	4	12	20	78	128
8	<2	<4	3	<10	98	1024
	64	128	24	160	423	8192
9	<2	<4	2	<10	195	256
	<2	8	24	80	444	512
10	<2	<4	<2	<10	43	32
	64	64	96	>160	2292	2048

TABLE III. Reproducibility of the Radioimmunoprecipitation Test.

Human serum	Reciprocal of antibody titer		
	1	2	3
1	2048	2048	4096
2	4096	4096	4096
3	1024	1024	512
4	1024	2048	1024

In a blocking experiment purified membrane glycolipids of *M. pneumoniae* reduced the titer of a human antiserum 4-fold (Fig. 3). These results indicate that the RIP test measures antibody to membrane glycolipids. The data in Fig. 3 also indicates that a proportion of RIP antibody is directed toward other membrane components.

Lipids of *M. pneumoniae, M. neurolyticum* and *M. fermentans* which were extracted by chloroform methanol (2:1, v/v) showed high complement fixing activity with homologous antiserum. Cross reaction was observed to a slight degree only with *M. neurolyticum* and rabbit *M. pneumoniae* hyperimmune serum (Table V). When these lipids were studied for their capacity to block *M. pneumoniae* antibody in human serum a significant blocking effect was seen only with *M. pneumoniae* lipids although *M. neurolyticum* lipids showed a slight blocking effect with some sera. Purified membrane glycolipids of *M. pneumoniae* were effective in blocking antibody in all sera. This was observed with sera from volunteers infected intranasally with *M. pneumoniae,* sera from

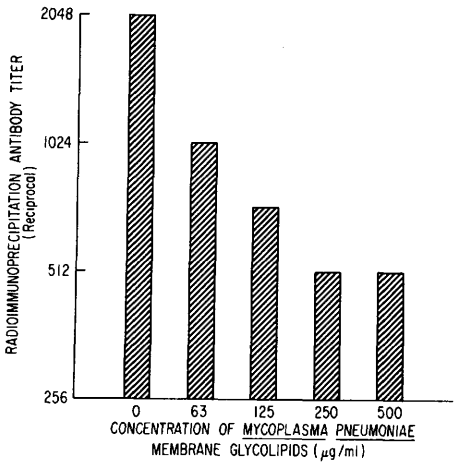

FIG. 3. Blocking of *M. pneumoniae* RIP antibody in a human convalescent serum by membrane glycolipids of the organism.

children less than 5 yr of age and sera collected from healthy marine recruits several months before development of pneumonia caused by *M. pneumoniae* (Table VI).

Discussion. A radioimmunoprecipitation test for the detection of antibody to microorganisms has first been developed by Gerloff, Hoyer and McLaren (24) for type 2 poliovirus. This test proved to be highly sensitive without loss of specificity. The development of a similar test procedure for *M. pneumoniae* was made possible by the observation of Razin *et al.* (8) that lipid precursors in the growth medium were incorporated into the mycoplasma membrane. The method of labeling *M. pneumoniae* membrane lipids with ^{14}C-fatty acids rather than using

TABLE IV. Specificity of the Radioimmunoprecipitation Test for Detection of Mycoplasma Antibody.

Infection with indicated mycoplasma	Volunteer no.	Reciprocal of radioimmunoprecipitation antibody titer ($\times 10^3$) in human serum			
		M. pneumoniae		*M. hominis*	
		Pre-inoculation	Post-inoculation	Pre-inoculation	Post-inoculation
M. pneumoniae	1	8.2	67.5	0.5	0.5
Strain PI 1428	2	8.2	>67.5	1.0	2.0
M. hominis	3	8.2	8.2	1.0	8.2
Strain DC 63	4	16.4	8.2	0.5	8.2

TABLE V. Reactions of Lipid Antigens and Homologous and Heterologous Mycoplasma Antisera.

Lipid antigen[a]	Highest antigen dilution (reciprocal) which fixed 1.8 units of complement with indicated rabbit hyperimmune serum		
	M. pneumoniae	*M. neurolyticum*	*M. fermentans*
M. pneumoniae	2048	<4	<4
M. neurolyticum	16	4096	<4
M. fermentans	<4	<4	1024

[a] Extracted from organisms by chloroform–methanol (2:1, v/v).

iodine isotopes to label the protein of the organisms was chosen for two reasons. First it was recently shown that membrane glycolipids of *M. pneumoniae* are the major antigens in conventional serologic reactions (22, 23). Second the long half-life of carbon-14 permits the use of a single pool of radioactive antigen over a long time period thus making standardization of new antigen preparations unnecessary. As in the mycoplasmacidal test it was necessary to use a homogeneous monodispersed suspension of filtered organisms as antigen.

The radioimmunoprecipitation test proved to be considerably more sensitive than conventional methods and slightly more sensitive than the MCT for the detection of antibody to *M. pneumoniae*. This increase in sensitivity was not accompanied by an apparent loss in specificity. The membrane glycolipids of *M. pneumoniae* appeared to be important sites for binding of RIP antibody to the organism. Thus, purified membrane glycolipids were capable of blocking 75% or more of the RIP antibody activity in human sera while the crude lipids of the organism blocked 50–80% of antibody activity. The crude lipids of *M. neurolyticum* were less effective in blocking RIP antibody for *M. pneumoniae* in human sera, while the lipids of *M. fermentans* were without effect. These findings suggest that the antibodies which were detected in human serum by the RIP method were induced by prior experience with the antigens of *M. pneumoniae*. Of course, one must consider the possibility that much of the antibody detected by RIP was stimulated by cross-reactive glycolipids from other sources.

Complement or complement components do not seem to be necessary for the binding of antibody by radiolabeled *M. pneumoniae*. For this reason the RIP method offers the possibility of measuring complement inde-

TABLE VI. Blocking of *M. pneumoniae* Radioimmunoprecipitation (RIP) Antibody by Lipid Antigens.

Human serum	RIP antibody titer (reciprocal)	Fold decrease following addition of indicated material			
		Purified *M. pneumoniae* glycolipids (100 μg/ml)	Total lipids (500 μg/ml)		
			M. pneumoniae	*M. neurolyticum*	*M. fermentans*
Volunteer infected with *M. pneumoniae*	(a) 2048	4	2	None	None
	(b) 4096	4	2	None	None
Marine recruit before development of *M. pneumoniae* pneumonia	(a) 1024	8	5.3	2	None
	(b) 512	8	4	2	None
Normal child 3 yr	2048	16	4	2	None
Normal child 5 yr	1024	8	4	2	None

pendent antibodies, especially those of the IgA class. These antibodies are of potential interest in *M. pneumoniae* infection because IgA is the predominant functional immunoglobulin in local secretions. Previous attempts to demonstrate antibody to *M. pneumoniae* in nasal or bronchial secretions of persons infected with the organism had either failed or yielded conflicting results (26–29). In a study in progress we have found that a high proportion of volunteers inoculated intranasally with *M. pneumoniae* developed a fourfold or greater rise in RIP IgA antibody in nasal secretions. The significance of local antibody to *M. pneumoniae* in the disease caused by this organism is currently under investigation.

Summary. A radioimmunoprecipitation (RIP) test for detection of antibody to *M. pneumoniae* was developed. The test was performed by using microtiter equipment thus combining ease of performance and the need for small amounts of reagents. Divalent cations did not enhance the RIP antibody titer of human sera to *M. pneumoniae* indicating that complement or complement components were not fixed. Membrane glycolipids appeared to be the major although not the only antigens involved in the RIP reaction. The RIP method was slightly more sensitive than the complement dependent mycoplasmacidal antibody assay and considerably more sensitive than the standard serologic techniques used for measurement of antibody to *M. pneumoniae*.

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