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Received June 3, 1968. P.S.E.B.M., 1968, Vol. 129.

Immunodiffusion Reactions between Human Sera and *Mycoplasma pneumoniae** (33331)

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(Introduced by H. G. Cramblett)

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Mycoplasma pneumoniae is an important respiratory tract pathogen of man. Although this microorganism can be present in individuals who are asymptomatic, its effects may range from mild respiratory disease to primary atypical pneumonia characterized by the development of cold hemagglutinins (1, 2).

Current procedures for isolation and identification of *M. pneumoniae* require the use of complex culture media and prolonged incubation periods (3, 4). Consequently, many investigators have relied upon serologic procedures to obtain evidence of *M. pneumoniae* infection. Tests used to detect *M. pneumoniae* antibody in human sera include complement-fixation, immunofluroescence, hemagglutination, and growth inhibition procedures (5, 6).

The tetrazolium reduction inhibition test (TRI) is a sensitive means of measuring growth inhibiting antibody against *M. pneumoniae*. If specific antibody is present, the organism does not grow and the tetrazolium dye indicator is not reduced. Growth inhibiting antibody has been correlated with resistance to illness caused by *M. pneumoniae* (7). Volunteers infected with *M. pneumoniae* were protected against illness when detectable levels of growth inhibiting antibody were present prior to challenge. In addition, growth inhibiting antibody at a level of 1:16 or greater prevented infection. Antibody responses to *M. pneumoniae* vaccines are evaluated frequently by the TRI test (8, 9). This test requires a 5–6 day incubation and can be invalidated by bacterial contamination or nonspecific inhibitors in the serum. Double diffusion gel precipitation (ID) techniques are not subject to these limitations.

Previously, Taylor-Robinson *et al.* (10) demonstrated ID reactions with several animal mycoplasmas and hyperimmune animal sera. However, they experienced difficulties in obtaining a satisfactory *M. pneumoniae* ID antigen. Even with improved methods for

* Supported by Public Health Service Research Contracts PH 43-65-562, PH 43-67-78 and PH 43-67-79 from the Vaccine Development Branch of the National Institute of Allergy and Infectious Diseases.

cultivating mycoplasmas (11), *M. pneumoniae* antigens reactive with animal immune sera could be obtained only after concentrating organisms 400- to 1000-fold (12). These antigens have not been used to demonstrate human antibody to *M. pneumoniae.* We have prepared a *M. pneumoniae* antigen that can be utilized in an ID test for detecting human antibody.

Materials and Methods. Medium. The growth medium was developed in studies on the immunogenicity of *M. pneumoniae* preparations (13). Its composition per liter was: glucose, 5.0 g; Difco PPLO broth dehydrated, 7.4 g; fresh yeast extract, 50 ml (Microbiological Associates, Cat. no. 26-230); PPLO serum fraction, 50 ml (Microbiological Associates, Cat. no. 14-447); Eagle's Minimum Essential Medium, 4.8 g (Grand Island Biologicals, Cat. no. F-11); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 11.9 g (Calbiochem. Cat. no. 391338), phenol red 2.0 mg, thallium acetate 25 mg; penicillin G, 500,000 units. The pH was adjusted to 7.4 with NaOH.

Preparation of antigen. To produce an antigen, 0.1 ml of a suspension of *M. pneumoniae* strain FH (three hundred twenty-fifth passage level) was inoculated into 25 ml of medium contained in a 6 oz prescription bottle. The culture was incubated at 37° with the bottle in the horizontal position. The broth was decanted after 4 days and the layer of organisms attached to the glass was washed four times with 10 ml of phosphate buffered saline, pH 7.3. Organisms were removed from the glass by scraping, and resuspended in 0.4 ml of distilled H_2O. Mycoplasmas grown in several prescription bottles were treated in a similar manner and pooled to form a standard antigen preparation which was then frozen and thawed 10 times. Except in specificity studies described later, aliquots of this standard preparation were used in all ID tests. Although not employed in this study, *M. pneumoniae* antigens reactive in ID can be made without freezing and thawing. Antigen preparations have been reactive in ID tests after 6 months storage at 4°.

Sera. Serial serum specimens from marine recruits suspected of having *M. pneumoniae* infection were supplied by Richard R. Gutekunst, Camp LeJuene, North Carolina. The recruit population has been described (9). Sera from volunteers were received from William J. Mogabgab of Tulane University. Volunteers had been injected with inactivated *M. pneumoniae* vaccine lot 3101-J6-SA produced by Charles Pfizer Company, Terre Haute, Indiana. Animal immune sera were prepared as described previously (10). Sera were not heat inactivated prior to use.

Tetrazolium reduction inhibition tests (TRI). TRI tests were performed as described by Senterfit and Jensen (14).

Cardiolipin-albumin reagent. A cardiolipin-albumin reagent was prepared by J. Dennis Pollack of Ohio State University. Bovine cardiolipin was obtained from Applied Science Corporation, State College, Pa., and bovine albumin, Fraction V Powder from Pentex, Inc., Kankakee, Ill. Cardiolipin (lot 769-24), 2.5 mg., in 0.1 ml chloroform was added to 1.0 ml of filtered 5.0% (w/v) bovine albumin, Fraction V Powder, fatty acid poor (lot 17) in 0.15 *M* phosphate buffered saline (p*H* 7.2). Nitrogen was aseptically passed through the two phase system at room temperature for approximately 30 min until no odor of chloroform could be detected. The resultant turbid suspension was used directly or stored at 4°.

Double diffusion gel precipitation (ID). A micromethod for ID was employed using 0.6% agarose prepared in 0.15 *M NaCl* (15). Wells for antigen and human serum were 4 mm in diameter and contained 45 μl of reagent. When animal immune sera were used, wells were of 3-mm diameter and contained 25 μl. Diffusion distance between wells was 2–3 mm.

Staining of ID reactions. The procedures for preparing and staining ID reactions with Sudan black for lipid and triple stain for protein are as described by Crowle (16).

Results. Reactivity of M. pneumoniae *antigen.* When human convalescent phase sera from patients infected with *M. pneumoniae* were reacted with the *M. pneumoniae* antigen in ID, one or more precipitation bands were formed with each serum. Lines developed be-

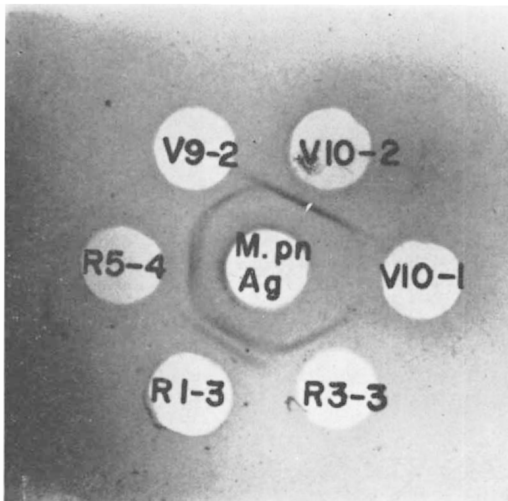

FIG. 1. The ID reactions between *M. pneumoniae* antigen and sera from vaccinees and naturally infected marine recruits. The center well contains *M. pneumoniae* antigen, strain DC 65-2053; the peripheral wells contain convalescent phase sera from vaccinees (V9 and V10) and recruits (R3 and R5); the number after the hyphen indicates the corresponding serum specimen; ID reactions unstained.

tween 5 and 16 hr of incubation at 33°. At least one band was common to all sera, as shown by reactions of identity, when antigen was placed in the central well and sera in adjacent peripheral wells (illustrated in Fig. 1). The formation of lines indicated that the *M. pneumoniae* antigen was reactive with human sera. Only those *M pneumoniae* antigen preparations which formed the common band with a standard convalescent phase serum were considered reactive.

Demonstration of sero-conversion. To determine the usefulness of the ID procedure in demonstrating sero-conversion, we tested serum specimens from 32 marine recruits selected because of TRI antibody responses to *M. pneumoniae* (Table I). These sera had been collected at monthly intervals from a group of marines training at Parris Island, South Carolina. During this period, 31 recruits had evidence of infection with *M. pneumoniae* as was indicated by a significant rise in TRI antibody; *M pneumoniae* had been isolated from 16 of the recruits, including one (R32) who had not shown TRI antibody conversion.

Within 1 month, 3 of the 32 recruits (R6, R27, and R28) had shown a fourfold or greater TRI antibody response. In ID, these sera elicited one to two precipitation bands, whereas the initial sera from these recruits did not react. The development of bands or an increase in the number of bands was interpreted by us as evidence of serologic conversion. A fourth recruit, (R3) had shown antibody conversion by the TRI test (titer of 16) with negative results by ID. In a repeat of the TRI test the titer was 2, thus removing him from the group showing conversion. The TRI test was repeated on other serum specimens in which the TRI and ID procedures did not yield comparable results (values from the retests are shown in brackets in Table I).

By the third serial bleeding, 2 months after the initial specimen, 18 of the 32 recruits (56%) had shown a fourfold or greater TRI antibody response. This same group of 18 plus a nineteenth demonstrated sero-conversion by gel diffusion (59%). With sera from final bleedings, 29 recruits had shown sero-conversion in both TRI and ID tests. Of the 3 remaining recruits, 1 showed conversion by TRI alone (R12), 1 by gel alone (R32), and the third (R11), after repeat of the TRI test, did not demonstrate conversion by either method.

An analysis of the data on the 32 recruits reveals that ID antibody was detected in every serum specimen which had a TRI titer of 16 or greater. Five of eight sera with titers of eight gave positive reactions in gel tests; whereas only one of four sera with a TRI titer of 4 was positive. ID bands were observed with only one serum which had a titer below 4.

Some serum specimens gave multiple lines in ID reactions. With the exception of one recruit (R9), the presence of more than one precipitation band was associated with a 32-fold or greater rise in antibody titer. Multiple bands were not produced with any serum having a titer less than 32. However, some serum specimens with titers as high as 2048 gave only a single ID line. Despite the

TABLE I. Antibody Response in Marine Recruits Infected with *M. pneumoniae* as Measured by Tetrazolium Reduction Inhibition (TRI) and Gel Diffusion.[a]

Recruit no.	TRI antibody titer (above) and no. of gel diffusion lines (below)				Recruit no.	TRI antibody titer (above) and no. of gel diffusion lines (below)			
	First serum	Second serum	Third serum	Fourth serum		First serum	Second serum	Third serum	Fourth serum
R 1	4	4	256	64	R 17	2	2	128	NA
	0	0	2	2		0	0	1	
R 2	<1	<1	1024	1024	R 18	2	2	2	128
	0	0	2	2		0	0	0	2
R 3	<1	16[2]	32	64	R 19	<1	1	1	512
	0	0	1	1		0	0	0	2
R 4	2	2	64	64	R 20	2	4	128	64
	0	0	2	2		0	0	1	3
R 5	2	2	256	128	R 21	<1	2	128	128
	0	0	3	3		0	0	2	2
R 6	2	8	8	8[64]	R 22	<1	<1	128	128
	0	1	1	2		0	0	2	2
R 7	8	8	4	256	R 23	1	1	<1	256
	0	0	0	1		0	0	0	1
R 8	<1	<1	<1	64	R 24	<1	<1	<1	>2048
	0	0	0	3		0	0	0	3
R 9	16	8	8	128	R 25	<1	<1	32	32
	1	1	1	2		0	0	1	2
R 10	4	8	4	256	R 26	<1	1	256	2048
	0	0	1	2		0	0	1	1
R 11	2	2	2	16[4]	R 27	<1	>2048	256	1024
	0	0	0	0		0	2	2	1
R 12	<1	<1	<1	32[8]	R 28	<1	64	64	64
	0	0	0	0		0	2	3	2
R 13	<1	<1	<1	512	R 29	2	2	2	256
	0	0	0	2		0	0	0	2
R 14	<1	<1	<1	512	R 30	<1	2	64	64
	0	0	0	1		0	0	2	2
R 15	2	2	32	NA	R 31	<1	2	128	128
	0	0	1			0	0	2	3
R 16	<1	2	64[8]	NA	R 32	4	4	4	4[2]
	0	0	1			0	0	0	1
Cumulative no. of recruits with a fourfold or greater rise in TRI antibody							3	18	30
Cumulative no. of recruits whose sera showed development of lines in gel diffusion							3	19	30

[a] Serum specimens were collected at monthly intervals after the first serum. A titer of less than one indicates that inhibition was not detected with undiluted serum. The numbers in brackets indicate results of a second TRI test and are the values used in cumulative totals. NA indicates that serum specimens were not available.

variation in the number of ID bands observed with different sera, reactions of identity indicated that one antibody was common to all positive sera.

Sera were also examined from individuals who had received an experimental *M. pneumoniae* vaccine (Table II). Three serial bleedings were available from each of 12 vaccinees. Three vaccinees (V3, V6, and V10) had TRI and ID antibody in their initial sera. Six vaccinees showed TRI antibody conversion at 1 month. Although all 6 demonstrated ID antibody at 1 month, only 5 can be considered as converting serologically. The sixth (V6) had ID antibody in the first serum and did not develop additional bands. With another vaccinee (V10), the converse was true; no rise was detected by TRI, but an additional band was formed. Data from the third bleeding reveal one more sero-conversion (V12) by ID only. Three vaccinees did not have TRI or ID antibody in the sera of any of their serial bleedings. Again, positive sera also produced a common band when reacted with *M. pneumoniae* antigen. As evidenced by reactions of identity (Fig. 1), the antibody involved was similar to the antibody detected in reactions with sera from naturally infected recruits.

Specificity of reactions. Although *M. pneumoniae* is antigenically the most distinct of the human mycoplasma species (10, 12), we performed the following experiments to establish the specificity of the ID reactions:

(i) Antigens of human mycoplasma species, *M. hominis, M. orale* type 1, *M. fermentans, M. salivarium,* our standard *M. pneumoniae,* the avian species *M. gallisepticum* and the rat species *M. pulmonis* produced ID bands with homologous rabbit immune serum. Except for *M. pneumoniae,* these antigens failed to form ID lines with human sera which had been shown previously to react with the standard antigen. All gel antigens were prepared from organisms grown in the same medium under the same conditions. Components of the mycoplasma growth medium, including the PPLO serum fraction and yeast extract, did not react with the human sera in ID.

(ii) Since *M. pneumoniae* is thought to contain a cardiolipin-like antigen (17), *M. pneumoniae* ID antigen was tested against 10 Wassermann positive sera. In the same test, we included in separate wells cardiolipin conjugated with bovine albumin, bovine albumin alone, and *M. pneumoniae* positive sera from recruits. No bands were observed in reactions between *M. pneumoniae* antigen and Wassermann sera. An ID line was formed between the cardiolipin-albumin preparation and 2 Wassermann positive sera adjacent to it. These same sera did not react with the albumin alone.

TABLE II. Comparison of TRI and Gel Diffusion Antibody Responses in Individuals Receiving an Experimental *M. pneumoniae* Vaccine.[a]

Volunteer no.	TRI antibody titer (above) and no. of gel diffusion lines (below)		
	First serum	Second serum	Third serum
V 1	<1	32	32
	0	1	2
V 2	<1	<1	<1
	0	0	0
V 3	32	64	64
	1	1	1
V 4	<1	16	4
	0	1	1
V 5	4	32	16
	0	1	1
V 6	16	256	128
	1	1	1
V 7	<1	<1	<1
	0	0	0
V 8	1	4	4
	0	1	1
V 9	2	8	8
	0	1	1
V 10	64	64	64
	1	2	2
V 11	<1	<1	<1
	0	0	0
V 12	<1	<1	1
	0	0	1

[a] Serum specimens were collected at 4- (second) and 7-week (third) intervals after the first serum. A titer of less than 1 indicates that inhibition was not detected with undiluted serum.

(iii) Sera from individuals who had no evidence of *M. pneumoniae* infection were tested in ID. This group included 10 patients with clinical diagnosis of upper respiratory tract infection, 1 with lower respiratory tract infection, 1 with myocarditis, and 5 healthy laboratory personnel. The standard *M. pneumoniae* antigen did not give ID bands with any of these sera.

(iv) In addition to the standard antigen produced from the FH strain, ID antigens were prepared from other *M. pneumoniae* isolates. One strain, PI 1428, was obtained from Robert M. Chanock at NIAID. It had been isolated on a medium of Hayflick's formula (18). The organism was subcultured for four passages on medium containing PPLO serum fraction. Another antigen was produced from *M. pneumoniae* strain DC 65-2053, an organism reisolated in our laboratory from the original clinical material sent to us by Dr. Jose Canchola of the Division of Biologic Standards at NIH. The organism was passaged once on medium of the Hayflick formula and then subcultured eight additional passages on the PPLO serum fraction medium already described, but without HEPES buffer.

Reactions were similar to those observed with the standard antigen (Fig. 1). However, in an ID reaction between DC 65-2053 antigen and the last serial bleeding from one of the recruits (R5), only one gel band was resolved. Three bands had been formed when this serum was reacted with the standard antigen (Table I). This variation has also been observed with different antigenic preparations of the FH strain. However, while the absolute number of bands varied, the antigen taking part in the formation of the common ID line was always present.

Staining of reactions. The common ID band is stained by both a lipid stain, Sudan black, and protein stains. Therefore, the antigen participating in the formation of the common band is probably lipoprotein. Additional bands, as seen in Fig. 1, accept protein dyes but not Sudan black.

Discussion. The data on the immune response of both naturally infected and vaccinated individuals indicate that the ID procedure is a sensitive means of detecting human antibody against *M. pneumoniae.* There is excellent agreement between results obtained by TRI and ID methods.

The degree of sensitivity of the ID test is somewhat surprising. Generally, double diffusion gel precipitation is thought to be an insensitive means for antibody assay. In contrast, the TRI test is considered one of the most sensitive of currently used serologic methods for detecting human antibody to *M. pneumoniae* (5). In our studies, the ID test detected antibody in every serum with a TRI titer of 16 or greater and in 63% of those with a titer of 8. Specific antibody was even detected in one serum with a TRI titer of 2.

Similar sensitivity in detecting antibody by double diffusion gel precipitation has been described for the enteroviruses (15). With these viruses, it has been suggested, because of the nature of the antigen involved, that the reaction was microagglutination rather than precipitation. In the present study, the physical nature of the mycoplasma antigen has not been established.

The sensitivity of the ID test could be increased further by taking advantage of the "recruiting" or "enhancement" phenomenon (16). Using this method, detection of antibody is facilitated by placing a strongly positive serum in the well adjacent to a serum which is suspected of having a low level of antibody. When recruiting was employed, some additional sera with TRI titers of 2 or less gave reactions by ID. Recruitment might be useful for screening individuals for volunteer studies, or in determining the degree of protection afforded by low levels of antibody.

Lemcke *et al.* (17) have reported gel diffusion reactions of partially purified carbohydrate preparations from *M. mycoides* with *M. pneumoniae* human convalescent phase sera. They were also able to demonstrate reactivity of a *M. pneumoniae* chloroform–methanol lipid extract with hyperimmune rabbit *M. pneumoniae* sera. The present study is the first report in which suspensions of *M. pneumoniae* organisms have been used in gel diffusion for the detection of human antibody.

At least one common antibody was found

in all positive sera. This antibody reacted with *M. pneumoniae* ID antigen to form a complex which accepted a lipid as well as a protein stain. Additional bands formed in reactions with some sera and antigen accepted only protein stain. Other investigators have speculated that the growth inhibiting antibody to *M. pneumoniae* is directed against an antigen that contains lipid (19). Since there is good correlation between antibodies detected in ID and TRI tests, the present findings support the assumption that a lipid component is involved in the formation of growth inhibiting antibody.

In addition to its specificity and sensitivity, the ID test is a rapid, easy method for the detection of human antibody to *M. pneumoniae*.

Summary. *M. pneumoniae* antibodies in human sera are detectable by double diffusion gel precipitation. These antibodies correlate with growth inhibiting antibody measured by the tetrazolium reduction inhibition test. All positive sera share a common precipitation band which appears to contain lipid.

We thank Diana Dunphy and Evelyn Blackman for technical assistance.

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Received June 10, 1968. P.S.E.B.M., 1968, Vol. 129.

Fluorescent Plaque Assay of Rubella Virus Infectivity (33332)

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The need for a rapid and precise plaque assay for quantitating rubella virus infectivity has long been recognized. Several plaque tests have been described earlier (1–9), but thus far no single infectivity assay has been applied with uniform ease and advantage from one laboratory to another. Most of these procedures require a relatively long period of cell viability under agar and the use of virus adapted to the assay cell system by serial passage.

In this report a fluorescent focus assay