Relationship of *Mycoplasma pneumoniae* to Other Human *Mycoplasma* Species Studied by Gel Diffusion

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ABSTRACT

TAYLOR-ROBINSON, DAVID (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), OTAKAR SOBĚSLAVSKÝ, AND ROBERT M. CHANOCK. Relationship of *Mycoplasma pneumoniae* to other human *Mycoplasma* species studied by gel diffusion. J. Bacteriol. 90:1432-1437. 1965.—Conditions are presented for the production of four lines of precipitate between *Mycoplasma pneumoniae* antigen and homologous hyperimmune rabbit serum in double diffusion in agar. The specificity of the reaction was shown by the fact that *M. pneumoniae* antigen did not react with antisera to the other human mycoplasma species, nor did *M. pneumoniae* antiserum produce lines with antigens prepared from the other human mycoplasmas. In addition, there was no reduction in the number or intensity of precipitation lines after absorption of *M. pneumoniae* antiserum with heterotypic mycoplasma antigens, or after absorption of heterotypic mycoplasma antisera with *M. pneumoniae* antigen. These findings indicate that, of the human mycoplasma species so far studied, *M. pneumoniae* is antigenically the most distinct.

Mycoplasma pneumoniae, the agent of primary atypical pneumonia, is one of several mycoplasma species which have been isolated from man. Each of these species has been shown to be distinct in growth inhibition tests (Clyde, 1964; Taylor-Robinson et al., 1964; Taylor-Robinson, Fox, and Chanock, 1965), but relationships between the species other than *M. pneumoniae* have been demonstrated in indirect hemagglutination, complement-fixation, and agar gel-diffusion tests (Taylor-Robinson et al., 1965). The Ouchterlony technique of double diffusion in agar, although not a sensitive method for the quantitative measurement of antibody, has proved to be of great value in the study of antigenic relationships between mycoplasma species. However, previous gel-diffusion studies of *M. pneumoniae* (Taylor-Robinson et al., 1963) were unsatisfactory, since only one weak band of precipitate formed after incubation of homologous antigen and antiserum for 48 hr. The preparation of more reactive *M. pneumoniae* antigens and antisera has permitted the relationships

between this mycoplasma species and other human species to be studied in greater detail in agar double-diffusion tests. The results of this study are presented in this communication.

MATERIALS AND METHODS

Organisms. *M. pneumoniae* was a laboratory-adapted strain (FH) grown on artificial medium for over 50 passages (Chanock, Hayflick, and Barile, 1962). The other human mycoplasma strains have been described previously (Taylor-Robinson et al., 1965).

Hyperimmune rabbit sera. Antisera to the prototype mycoplasma strains were prepared in rabbits as described in detail previously (Taylor-Robinson et al., 1963). Briefly, mycoplasma organisms were grown in rabbit infusion broth supplemented with rabbit serum, and 10- or 20-fold concentrates were used to inoculate rabbits. *M. pneumoniae* antisera 12324 and 12391 were prepared in this way. The other antisera to *M. pneumoniae*, 10692 and 10934, were prepared by inoculating rabbits with infected suspensions of chick embryo lung.

Absorption of rabbit antisera. Absorption was performed by mixing the serum with an equal quantity of homologous or heterologous antigen and incubating the mixture at 37 C for 2 hr with occasional agitation. The mixture was then centrifuged at 600 × g for 10 min, and the supernatant fluid was used in the test.

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Serological techniques. The methods used for complement fixation (CF), indirect hemagglutination (IHA), and growth inhibition have been described previously (Taylor-Robinson et al., 1964). The tetrazolium reduction inhibition (TRI) test was a modification of that described by Jensen (1964). Gel antigens for all mycoplasma species, except *M. pneumoniae*, were prepared from organisms which were grown in 400-ml amounts of standard PPLO broth (Taylor-Robinson et al., 1963) in screw-capped bottles. The cultures were incubated at 36 C for 10 days, and the media were then centrifuged at $44,000 \times g$ for 30 min in a model L (21 rotor) Spinco Ultracentrifuge; the deposits were resuspended in distilled water to achieve a 400-fold concentration. *M. pneumoniae* was grown in 1-liter amounts of standard broth medium with 1% glucose contained as a shallow layer in Provitsky bottles stoppered with cotton-wool. The medium was incubated for 6 to 8 days, and after centrifugation the deposit was resuspended in distilled water to achieve a 400- to 800-fold concentration. The antigen concentrates were frozen and thawed 10 times or sonically treated at 10 kc for 60 min and then stored at -20 C. Control antigen prepared from uninoculated broth was treated in the same way. Rabbit antisera were used without heat inactivation. Tests were performed in agar gel contained in plastic plates as prepared by Hyland Laboratories, Los Angeles, Calif. When a reservoir was filled more than once, the refilling was performed as the fluid level was approaching the bottom of the reservoir; this was usually less

TABLE 1. Results of gel-diffusion studies with rabbit antisera to *Mycoplasma pneumoniae* and the reactivity of these sera in other serological tests

Rabbit antiserum* no.	Precipitation lines		Size of zone in growth inhibition disc test	Reciprocal of antiserum titer in†		
	First ap- peared at	No. at 24 hr		TRI	CF	IHA
	<i>hr</i>					
10692	2	2	9	2,560	1,024	2,048
10934	8	2	9	2,560	2,048	2,048
12324a‡	1	2	10	20,480	1,024	1,024
12324b	—	0	6	640	256	512
12324c	1	2	10	20,480	1,024	2,048
12391	1	2	6	640	256	2,048

* Antisera 10692 and 10934 were prepared by inoculating rabbits with infected suspensions of chick-embryo lung. Antisera 12324 and 12391 were prepared by inoculating rabbits with organisms grown in rabbit infusion broth medium.

† TRI, tetrazolium reduction inhibition; CF, complement fixation; IHA, indirect hemagglutination.

‡ Designations a, b, and c refer to different bleedings of the same rabbit.

TABLE 2. Effect on the number of precipitation lines of (i) refilling the reservoirs and (ii) diluting the antigen and antiserum

Serum reservoirs, no. of times filled with indicated serum dilution		Antigen reservoirs; no. of times filled with indicated antigen dilution						
		Filled twice	Filled once					
			Undiluted	2-fold	4-fold	8-fold	16-fold	32-fold
Filled twice	Undiluted	3*	3	3	3	2	1	0
Filled once	Undiluted	3	2	2	2	1	1	0
	2-fold	3	2	1	0	0	0	0
	4-fold	1	1	1	0	0	0	0
	8-fold	0	0	0	0	0	0	0

* Number of precipitation lines which developed.

than 2 hr after the initial filling. After the antigen-antibody reactions were considered to be complete, the agar was removed from the plastic container and placed on a glass microscope slide in preparation for photography.

RESULTS

Antigen and the production of precipitation lines. Previously we reported (Taylor-Robinson et al., 1963) the production of one precipitation line with an antigen concentrated 400-fold and a rabbit antiserum (no. 10692). This line appeared in 48 hr at 34 C. Further experiments were performed with the same rabbit antiserum and other more concentrated antigens which were prepared from broth cultures containing approximately 10^7 to 10^8 colony-forming units per ml. These antigens concentrated 600- to 800-fold produced better results, since two precipitation lines appeared. However, they were of poor intensity. After the production of another rabbit antiserum (no. 12324), gel-diffusion studies were performed with the same antigens; precipitation lines of much greater intensity than those produced by antiserum 10692 developed within a few hours with all these antigen preparations. The best defined lines, two to three in number, occurred with the 800-fold concentrated antigen, and no difference in line production was noted between the antigen frozen and thawed 10 times or that sonically treated at 10 kc for 60 min. Subsequently, the 800-fold concentrated antigen was used to test other rabbit antisera.

Antiserum and the production of precipitation lines. As shown in Table 1, five of the six antisera which were tested produced lines, although these differed in the time of their appearance and in

their intensity. Serum 10692 produced a line in 2 hr, and eventually two lines occurred but they were very poor in intensity. With three of the sera, 12324a, 12324c, and 12391, the first line appeared within 1 hr after the test was initiated. However, serum 12324a was of small quantity and insufficient for many tests, and serum 12391 produced a marked nonspecific halo which obscured the specific lines. Serum 12324c also produced a halo which was not so marked, and since the specific lines produced by it were of almost equal intensity to those produced by the other antisera it was used for most future experiments. All those antisera that had high levels of TRI, CF, and IHA antibody and that produced wide zones of growth inhibition also contained precipitating antibody which could be detected by the gel-diffusion technique. The best correlation between precipitating antibody and antibody measurable by other techniques was that observed with IHA. Antiserum 12324b which had the lowest titer of IHA antibody did not react in gel diffusion. Precipitating antibody did not correlate as well with antibody measurable by TRI and CF; antiserum 12324b did not react in the gel-diffusion test, whereas antiserum 12391, which had the same titer of TRI and CF antibody, produced two precipitation lines.

Refilling of reservoirs with antigen and antiserum and dilution of these materials. Refilling of the reservoirs with undiluted antigen and antiserum 12324c increased not only the intensity of the precipitation lines but also their number

(Table 2). In this particular experiment three lines were observed, although, in most other experiments in which the reservoirs were filled twice, four lines were observed. Even minimal dilution of the reagents decreased both the intensity and number of lines; the serum could not be diluted more than twofold and the antigen more than fourfold without a decrease in the number of lines.

Order of addition of antigen and antibody to reservoirs. In several experiments in which the reservoirs were filled once, the addition of antigen at the same time as, or 30 to 45 min before, the addition of antiserum resulted in the appearance of three or four lines of precipitate. On the other hand, the addition of serum before antigen resulted in the appearance of only two lines, presumably due to a nonseparation of lines.

Distance between antigen and antiserum reservoirs. In preliminary experiments in which antigens, concentrated 400-fold, and antisera were placed in adjacent reservoirs with a center-to-center distance of 5 mm, two precipitation lines were observed. Three lines were seen when the reservoirs were 4 mm apart.

Temperature of incubation. Antigen concentrated 800-fold and antiserum 12324a were incubated at 39 C, 34 C, and at room temperature. There was no marked difference in the rate of appearance of lines or in their intensity at any of these temperatures. An incubation temperature of 34 C was used routinely.

The optimal conditions for the formation of precipitation lines by *M. pneumoniae* antigen and antiserum may be summarized as follows: (i) antigen prepared from culture containing 10^7 or more colony-forming units per ml, concentrated 800-fold, either sonically treated at 10 kc for 60 min or frozen and thawed 10 times; (ii) undiluted antigen and antiserum used and reservoirs filled twice; (iii) antigen added at the same time as, or before, the antiserum; (iv) reservoir center-to-center distance, 4 mm. Such precipitation lines are shown in Fig. 1 and 2.

Control materials. Control antigen, concentrated 800-fold, was prepared in the same way as *M. pneumoniae* antigen, except that the standard PPLO broth was not inoculated with the organism. When this control antigen was tested by gel diffusion against all the *M. pneumoniae* rabbit antisera except 10692, no lines of precipitate were observed after 66 hr at 34 C. Preinoculation rabbit sera tested against the *M. pneumoniae* antigen concentrated 800-fold failed to produce precipitation lines after 24 hr at 34 C.

M. pneumoniae antigen tested against rabbit antisera to heterologous mycoplasma species. The

FIG. 1. Reaction between *Mycoplasma pneumoniae* antigen (PN.a) and homologous antiserum (12324c), and between the same antigen and *M. salivarium* antiserum (SAL.S) and *M. hominis* type 2 antiserum (H-2.S). Cups filled twice.

FIG. 2. Reaction between *Mycoplasma pneumoniae* antigen (PN.a) and its homologous antiserum unabsorbed (12324c + saline), or absorbed with *M. pneumoniae* antigen (12324c + PN.a), *M. hominis* type 1 antigen (12324c + H-1.a), or *M. orale* antigen (12324c + OR.a). Central cup filled twice and peripheral cups three times.

M. pneumoniae antigen was tested with rabbit antisera prepared against *M. hominis* type 1 (strain DC63), *M. hominis* type 2 (strain Campo), *M. fermentans*, *M. salivarium*, *M. orale*, and a recently identified human species isolated from the oropharynx (Taylor-Robinson et al., 1965). The reservoirs were filled twice and incubation was performed at 34 C for 24 hr in one experiment and 36 hr in another. No lines of precipitate occurred with antisera to *M. hominis* types 1 and 2, *M. fermentans*, and *M. salivarium*. Fig. 1 shows the presence of four precipitation lines between the central reservoir containing 800-fold concentrated *M. pneumoniae* antigen and a peripheral reservoir containing homologous rabbit antiserum (12324c). Rabbit antisera to *M. salivarium* and *M. hominis* type 2 did not produce lines of precipitate, although in other tests they were reactive with their homologous antigens. In another pattern, nonspecific halo formation around reservoirs filled with antisera to *M. orale* and the newly recognized serotype (strain DC938) obscured the presence, if any, of faint precipitation lines. However, antisera to three other strains (DC1428, DC1600, CH20247) of the new serotype did not produce halos, and precipitation lines were not observed.

Tests with antisera prepared against heterologous mycoplasma species absorbed with *M. pneumoniae* antigen. To study more fully the relationship between *M. pneumoniae* and the other

human species, the following serum-absorption experiments were performed. *M. pneumoniae* antiserum 12324c was absorbed with *M. pneumoniae* antigen concentrated 800-fold; this absorbed antiserum did not produce precipitation lines with the homologous antigen, even when the antigen reservoirs were filled twice and the serum reservoirs were filled three times (Fig. 2). Of course, since the serum was diluted twofold, filling the serum reservoirs three times was equivalent to filling them 1.5 times with undiluted serum. After the successful absorption of *M. pneumoniae* antiserum by its homologous antigen, antisera to the heterologous human mycoplasma species were absorbed with the same *M. pneumoniae* antigen in the same manner. Then, both these absorbed and unabsorbed antisera were tested against their respective homologous antigens. As an example, Fig. 3 shows the lines produced between *M. fermentans* antigen and its homologous antiserum unabsorbed or absorbed with homologous, "control," or *M. pneumoniae* antigen. Five lines formed between *M. fermentans* antigen and unabsorbed rabbit antiserum. These lines did not form when homologous antigen was used to absorb the

FIG. 3. Reaction between *Mycoplasma fermentans* antigen (FERM.a) in the central cup and its homologous antiserum unabsorbed (FERM.S + Saline), or absorbed with homologous antigen (FERM.S + FERM.a), control antigen (FERM.S + CONT.a), or *M. pneumoniae* antigen (FERM.S + PN.a). Central cup filled once and peripheral cups twice.

FIG. 4. (A and B) Reactions between *Mycoplasma salivarium* antigen (SAL. a) and its homologous antiserum (SAL. S), and between the same antigen and *M. pneumoniae* antisera 10692 and 12324c. Cups filled once.

antiserum. No reduction in the number and intensity of lines was observed with the antiserum absorbed with control or *M. pneumoniae* antigen. Likewise, no reduction in the number and intensity of lines was observed when the other heterotypic antigens were tested against their antisera absorbed with *M. pneumoniae* antigen.

Heterotypic human mycoplasma antigens tested against rabbit antiserum to M. pneumoniae. Antigens of each of the six human mycoplasma species other than *M. pneumoniae* produced four to seven precipitation lines when placed in reservoirs adjacent to homologous rabbit antiserum. *M. pneumoniae* antisera 10692 and 12324c were tested in the same gel patterns against the heterotypic antigens, and no precipitation lines were observed. As an example, the reaction between *M. salivarium* antigen and *M. pneumoniae* antiserum is presented (Fig. 4A and B). At least four lines formed between *M. salivarium* antigen and its homologous rabbit antiserum. Although marked halos formed around reservoirs containing *M. pneumoniae* rabbit antisera 10692 and 12324c, no precipitation lines appeared between these antisera and the *M. salivarium* antigen (Fig. 4A). Occasionally, an antiserum which does not produce precipitation lines will do so when placed in a reservoir adjacent to a more potent antiserum, i.e., a "recruitment" effect. However, no evidence of such an effect

was observed when the *M. pneumoniae* antisera were placed next to the *M. salivarium* antiserum (Fig. 4B). A recruitment effect was not observed in tests with any of the other heterologous antigens.

Tests with M. pneumoniae antiserum absorbed with the heterotypic human mycoplasma antigens. Evidence for a weak antigenic relationship between *M. pneumoniae* and the other human mycoplasma species was sought in the following experiments. Heterotypic rabbit antisera were absorbed with their respective antigens, and in subsequent gel-diffusion tests these absorbed antisera failed to produce lines of precipitate with their respective antigens. The same antigens were then used to absorb *M. pneumoniae* rabbit antiserum 12324c. No difference in the time of appearance, the intensity, or the number of lines was observed when *M. pneumoniae* antiserum absorbed with different antigens was compared with the unabsorbed antiserum in the same test. A typical example of such a test is shown in Fig. 2. The appearance of more than four lines was due to a nonspecific halo which surrounded each serum reservoir. This was particularly evident around the reservoir containing 12324c serum and saline and may be seen also in Fig. 1 and 4. However, the formation of four specific precipitation lines by *M. pneumoniae* antiserum

was unaffected by its absorption with *M. hominis* type 1 or *M. orale* antigens.

DISCUSSION

Under the conditions that we have outlined, four precipitation lines were observed when *M. pneumoniae* antigen and specific rabbit antiserum were tested by gel diffusion. The number of organisms (colony-forming units) in broth suspensions used for the preparation of gel antigens in these experiments was 100- to 1,000-fold greater than in suspensions used in previous experiments, in which we observed only one line after a prolonged period of incubation. Thus, the use of a more concentrated antigen and also a more potent antiserum accounts for the ability to produce lines in contrast to previous unsatisfactory results. Dilution of either the antigen or antiserum was accompanied by a reduction in the number of lines. When reservoirs were filled once, two lines were observed, and in almost all experiments in which the reservoirs were refilled four lines were seen. The possibility that the increase in the number of lines was due to multiple lines of a single precipitate was considered. This might occur if the interval between the initial filling and the refilling was so prolonged that the initial fluid had completely diffused. However, this was not so, diffusion being one continuous event. Furthermore, very occasionally four lines were observed in tests in which the reservoirs had been filled only once.

The relative difficulty of producing lines of precipitate with *M. pneumoniae* antigen and homologous antiserum contrasts with the ease of producing lines of precipitate with the other human mycoplasma species and their antisera (Taylor-Robinson et al., 1963; Lemeke, 1965). The reason for this difference is not understood but is probably a function of the *M. pneumoniae* organism rather than its antiserum. In serological reactions other than precipitation, the *M. pneumoniae* antiserum has a reactivity with its homologous antigen comparable to the reactivity of antisera to other human mycoplasma species with their respective antigens.

Previously we reported (Taylor-Robinson et al., 1963) that one line was produced in tests with *M. pneumoniae* antiserum and *M. hominis* type 1 and *M. salivarium* antigens. Repeated efforts to demonstrate a precipitation line with *M. hominis* type 1 or *M. salivarium* antigens and a *M. pneumoniae* antiserum of greater potency

have failed. With both of these antigens it is possible that the previous demonstration of a line was due to a "recruitment" effect or possibly a nonspecific effect. In the present experiments, attempts to produce precipitation lines with a *M. pneumoniae* antigen and antisera to the other human species or lines with heterotypic antigens and *M. pneumoniae* antisera have failed; likewise, absorption of heterotypic antisera by *M. pneumoniae* antigen or absorption of *M. pneumoniae* antiserum by heterotypic antigens failed to decrease the number or intensity of the precipitation lines. Since in previous gel-diffusion studies extensive cross-reactions have been observed between human mycoplasma species other than *M. pneumoniae* (Taylor-Robinson et al., 1963, 1964, 1965), the results now presented indicate that *M. pneumoniae* is the most antigenically distinct human mycoplasma species so far studied.

Double - diffusion precipitation provides another system for studying the antigenic structure of *M. pneumoniae*, especially in relation to the nature and antigenicity of the components responsible for line production. Such experiments are in progress.

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