SEROLOGICAL RELATIONSHIPS AMONG HUMAN MYCOPLASMAS AS SHOWN BY COMPLEMENT-FIXATION AND GEL DIFFUSION

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ABSTRACT

TAYLOR-ROBINSON, DAVID (National Institutes of Health, Bethesda, Md.), NORMAN L. SOMERSON, HORACE C. TURNER, AND ROBERT M. CHANOCK. Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. J. Bacteriol. **85**:1261–1273. 1963.—Antigenic relationships among human mycoplasmas were studied by complement-fixation and agar gel diffusion techniques. Four recognized human species *Mycoplasma hominis* type 1, *M. hominis* type 2, *M. salivarium*, and *M. fermentans* were antigenically distinct in these tests. In addition, *M. pneumoniae* (Eaton agent, the etiological agent of cold agglutinin-positive atypical pneumonia) was different from these four species. Although these species were distinct, evidence of shared antigenic components was obtained in complement-fixation and agar gel diffusion tests. Since rabbits were immunized with mycoplasmas grown in rabbit muscle infusion broth supplemented with rabbit serum or, in the case of *M. pneumoniae*, with infected chick embryo lung suspension, the possibility that the heterologous reactions resulted from antibody to growth medium components could be excluded. Four recent mycoplasma isolates from the oropharynx were analyzed, and three were shown to be closely related to *M. hominis* type 1, and the fourth was closely related to *M. salivarium*. Although the recent isolates could not be distinguished from the related "prototype" human species by complement-fixation, differences could be detected by the agar gel diffusion technique.

The finding that one species of mycoplasma, *Mycoplasma pneumoniae* (formerly called Eaton agent), causes respiratory disease in man has stimulated the search for other mycoplasmas (pleuropneumonia-like organisms; PPLO) which might cause such illness. In recent studies of human respiratory disease, a large number of PPLO strains have been recovered (Mufson, *unpublished data*; Organick, 1962). It is necessary to evaluate the serological methods for the classification of mycoplasma strains, so that these organisms may be identified and their etiological importance assessed.

It was our purpose to define the antigenic relationships among human mycoplasmas. The complement-fixation and agar gel diffusion techniques were used to compare recognized mycoplasma strains with recent isolates. Several distinct mycoplasma species were found to share antigens. In addition, the agar gel diffusion method permitted the differentiation of very closely related strains.

MATERIALS AND METHODS

Organisms. The mycoplasmas used in this study are listed in Table 1, and include four human strains defined by Edward and Freundt (1956). Strain DC63 was isolated from a patient with pneumonia (Mufson, *unpublished data*). The Botteicher (Botte) strain was obtained from a healthy Marine recruit. Strain V2785 was isolated from a human volunteer who had received *M. pneumoniae* 9 days prior to sampling; no isolations of *M. pneumoniae* were made from this individual on repeated sampling (Couch, *unpublished data*). *M. pneumoniae* was a laboratory-adapted strain (FH) grown on artificial medium for over 50 passages; it was originally isolated from material supplied by Liu (1957). The monkey isolate, simian 698, was cultured from the oropharynx of a *Cercopithicus* monkey.

Media. The medium used for the propagation and maintenance of the organisms was a modification of that reported by Chanock, Hayflick, and

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TABLE 1. *Source of Mycoplasma strains*

Strain	Recovered from	Source of culture (reference)
Recognized human strains*		
M. hominis type 1 (PG21)	Genital tract	Edward (Nicol and Edward, 1953)
M. hominis type 2 (Campo, PG 27)	Genital tract	Edward, originally from Dienes
M. salivarium (PG20)	Oropharynx	Edward (Nicol and Edward, 1953)
M. salivarium Buccal	Oropharynx	Lemcke and Kleineberger-Nobel (Card, 1959)
M. fermentans G (PG18)	Genital tract	Edward (Ruiter and Wentholt, 1953)
M. pneumoniae FH (Eaton)	Oropharynx	Liu (1957), and Chanock et al. (1962*b*)
Human isolates (unclassified)		
Mycoplasma Navel	Navel	Ruiter (*unpublished data*)
Mycoplasma DC63	Oropharynx	Mufson (*unpublished data*)
Mycoplasma Botte	Oropharynx	Mufson (*unpublished data*)
Mycoplasma V2785	Oropharynx	Mufson (*unpublished data*)
Mycoplasma DC585	Oropharynx	Mufson (*unpublished data*)
PPLO, simian strain 698	Oropharynx	Somerson and Coates (*unpublished data*)

* These strains have been classified by Edward and Freundt (1956); in a previous publication, the name *M. pneumoniae* was suggested for the Eaton agent (Chanock et al., *in press*).

Barile (1962*b*). This medium contained PPLO Agar (Difco), 7 parts; uninactivated horse serum, 2 parts; 25% yeast extract, 1 part. PPLO Agar was prepared in 70-ml quantities and sterilized by autoclaving. After cooling in a water bath at 56 C, the agar was supplemented with horse serum (Cappel Laboratories, West Chester, Pa.) and yeast extract. The yeast extract was prepared by adding 250 g of Fleischmann's type 20-40 dry yeast to 1 liter of distilled water and heating to boiling. This mixture was filtered through Whatman no. 1 paper and the pH adjusted to 8.0 with sodium hydroxide; it was then distributed in 10-ml samples and autoclaved. The preparation was stored at −20 C until used. Since *M. pneumoniae* and other PPLO strains may require prolonged incubation, we added 100,000 units of penicillin, 50 mg of amphotericin B, and 50 mg of thallium acetate per 100 ml of "complete" medium. To standardize growth conditions, this complete medium, together with inhibitors, was used for maintaining all strains. The PPLO broth contained the same constituents, but no agar.

The antigens for rabbit immunization and gel diffusion study were prepared from organisms grown in a rabbit infusion broth. Infusions were prepared by adding 8 ml of distilled water to 1.0 g of finely ground rabbit meat and heating to 100 C in an Arnold sterilizer. This infusion was filtered through gauze and then through Whatman filter paper. Peptone (1%; Difco) and 0.5% sodium chloride were added. After reheating to dissolve the peptone and salt, the pH was adjusted to about 7.8, and the preparation was sterilized by autoclaving. A cholesterol solution was prepared by dissolving 100 mg of cholesterol in a minimal quantity of absolute ethanol and adding the dissolved steroid to 100 ml of hot distilled water. Then, 2 ml of this solution were added to each 100 ml of rabbit infusion. A guinea pig infusion was prepared by adding 3 ml of distilled water to 1.0 g of finely ground guinea pig meat (bone not removed). Further preparation was similar to that of the rabbit infusion, except that cholesterol was not added. Rabbit and guinea pig infusions were enriched with 5% normal rabbit serum and 5% normal guinea pig serum, respectively. *M. pneumoniae* failed to grow in either the rabbit or guinea pig infusion broth; in this instance, the PPLO broth medium with horse serum and yeast extract was used for preparation of complement-fixing antigen as previously described (Chanock et al., 1962*a*).

Production of rabbit and guinea pig hyperimmune sera. To eliminate nonspecific serological cross reactions to broth medium constituents, rabbits were immunized with antigens grown in rabbit infusion broth. PPLO strains originally

grown and maintained in complete PPLO medium were subcultured for at least four passages in the rabbit infusion medium. For guinea pig immunization, a similar procedure was followed with organisms grown in the guinea pig infusion medium. The mycoplasma strains were grown under aerobic conditions, except for *M. salivarium* PG20, Buccal, DC585, and *M. fermentans* which were grown under an atmosphere of 5% CO_2-95% N_2. After incubation of the infusion broth media at 36 C for at least 8 to 10 days, all the cultures were centrifuged at 20,000 rev/min (Spinco 30L rotor) for 30 min. Each of the deposits was then resuspended in one-tenth the original volume with buffered saline (pH 7.2). Penicillin was added to the suspensions to avoid contamination.

A portion of this preparation was mixed with an equal quantity of Freund's complete adjuvant (Difco) and another portion mixed with incomplete adjuvant (Difco). Animals were inoculated intradermally at multiple sites with a total of 1.0 ml of antigen-complete adjuvant mixture. A 1.0-ml amount of incomplete adjuvant mixture was given by the same route 3 weeks later. After a further 3 weeks, the same dosage without adjuvant was given intraperitoneally and the animals were then bled 1 week later for antisera. Immunization of guinea pigs with organisms grown in guinea pig infusion broth was accomplished in the same manner, except that a 0.5-ml inoculum was used. Since *M. pneumoniae* did not grow in rabbit broth, rabbits were immunized by multiple subcutaneous inoculations of infected chick embryo lung suspension. In this instance, it is probable that the material used for immunization did not contain host-system components in common with the broth medium used to prepare complement-fixing antigens.

Complement-fixation technique. The micro complement-fixation procedure was that described by Sever (1962). Antigens were prepared from organisms grown in complete PPLO media (Difco PPLO broth with horse serum and yeast extract) and concentrated tenfold by centrifugation. A total of 8 units of antigen were used in all complement-fixation tests. The antigens and the rabbit antisera were heated at 56 C for 30 min before use.

Immunofluorescence technique. The PPLO colonies were transferred from agar plates to glass slides by a technique previously described (Clark, Fowler, and Brown, 1961). Normal or immune rabbit serum (diluted in 10% guinea pig complement) was added to the slides. Slides were then treated with a sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate (Progressive Laboratories, Bethesda, Md.). The fluorescein conjugate also contained bovine albumin conjugated with rhodamine (at a final dilution of 1:200) as a counterstain.

Gel diffusion technique. The organisms were grown in rabbit or guinea pig infusion, which was incubated at 36 C for 8 to 10 days. The suspension was then concentrated by centrifugation at 20,000 rev/min (Spinco 30 L rotor) for 30 min. The deposits from 25 ml of broth were resuspended in approximately 0.06 ml of distilled water. The concentrates were frozen and thawed six times and stored at −20 C. For controls, uninoculated broth infusions were processed in an identical manner. The method of agar diffusion was essentially that described by Ouchterlony (1948). The agar consisted of 1.5% "Ion Agar no. 2" (Oxoid, Oxo Limited, London, England) with Merthiolate (1:10,000). Amounts (5 ml) of the agar were poured into plastic petri dishes 5 cm in diameter. Wells were cut with a template consisting of six peripheral wells with a central reservoir (Fig. 1). Each reservoir was 2.4 mm in diameter and the edge was 3.2 mm from the edge of an adjacent reservoir. After reagents had been added to the cups, the dishes were incubated at 33 C in a humidified atmosphere. Accurate drawings of precipitation lines were made as soon as they appeared and later as the lines continued to develop. Photographs of the reactions were taken with the lines illuminated by oblique light. When there was no further line formation, the agar was washed in physiological saline for 2 to 3 days, removed from the petri dish, and allowed to dry onto a lantern slide at room temperature. The dried preparations were then stained with Sudan Black and azocarmine B (Uriel and Grabar, 1956).

RESULTS

Complement-fixation studies. Recognized mycoplasma strains and strains recently isolated from the oropharynx were tested with their corresponding antisera in cross complement-fixation reactions (Table 2). Strains DC63, V2785, and Botte, recent isolates from the human oropharynx, were more closely related to *M. hominis* type 1 (a

TABLE 2. *Relation of human mycoplasmas as shown by complement fixation*

Mycoplasma antigen (eight units)	Strain	Reciprocal of complement-fixing antibody titer with indicated rabbit serum[a]										
		M. hominis				*M. hominis* type 2 Campo	*M. salivarium*		*M. fermentans* G[b]	*M. pneumoniae* Eaton FH[c]	Navel[b]	Simian 698
		Type 1 PG21	Oral strains				PG20	Oral DC585				
			DC63	2785	Botte							
M. hominis type 1	PG21	*1,280*	320	2,560	2,560	40; 80	80	20	<20	<20	160	640
Oral strain	DC63	640	*640; 1,280*	640	640	40	40	<20	<20	<20	320	640
	2785	5,120	1,280	*2,560*	2,560	80	40	<20	<20	<20	<20	1,280
	Botte	2,560	320	1,280	*640*	160	40	<20	<20	<20	20	640
M. hominis type 2	Campo	80	<20	80	<20	*2,560*	160	80	<20	<20	<20	80
M. salivarium[d]	Buccal 1	80	40	320	80	80	*10,240*	2,560	<20	<20	160	320
Oral strain	DC585	80	40	160	160	160	5120	*2,560*	<40	80	160	320
M. fermentans	G	40	40	80	20	80	40	<20	*160*	<20	320	<40
M. pneumoniae	Eaton-FH	80	80	80	160	40	40	<20	<20	*2,560*	160; 320	160
Navel	—	40	40	<20	20	40	<20	20	<20	<20	*1,280*	<40
Simian	698	1,280	640	2,560	640	20	20	<20	<20	<20	<20	*1,280*

[a] Except for the rabbit sera against *M. fermentans* and *M. pneumoniae*, all immunizations were performed with mycoplasma suspensions grown in rabbit infusion broth supplemented with rabbit serum.

[b] Rabbit serum kindly supplied by R. Lemcke. Immunization was performed with mycoplasmas grown in beef infusion broth supplemented with rabbit serum; washed suspensions of such mycoplasmas were used to inoculate rabbits.

[c] Rabbit immunized with a lung suspension prepared from chick embryos infected with *M. pneumoniae*.

[d] *M. salivarium* PG20 and Buccal were indistinguishable by complement fixation; in this test, the Buccal antigen was used.

genital strain) then to *M. salivarium* (an oral strain). In fact, *M. hominis* type 1 and the three oral isolates were serologically indistinguishable from each other. The simian 698 strain was the only other organism which showed complete cross reactivity with the *M. hominis* type 1 strain. *M. hominis* type 2 appeared to be relatively specific. In a complement-fixation test not shown, *M. salivarium* PG20, *M. salivarium* Buccal, and DC585 were found to be identical. This group of strains was distinct from the other strains shown in Table 2. *M. fermentans*, *M. pneumoniae*, and the Navel strain also showed relative specificity.

Gel diffusion studies. We wished to determine whether strains that were indistinguishable in complement-fixation tests were, in fact, identical. We also wanted to clarify the relationship of strains that showed minor cross reactions by the complement-fixation technique. The gel diffusion technique was used for both purposes. Antigens obtained from strains grown in a rabbit infusion medium were used; this same antigenic material had been used to immunize rabbits for hyperimmune sera. Since *M. pneumoniae* did not grow in rabbit infusion, antigen for this organism was prepared in the complete PPLO broth medium, as mentioned earlier.

The rabbit hyperimmune sera produced lines of precipitation with homologous PPLO antigens grown in rabbit infusion medium. Concentrates of uninoculated rabbit infusion broth gave no precipitation bands with the hyperimmune sera; rabbit sera obtained before immunization did not react with the PPLO antigens prepared from rabbit infusion (Fig. 1). These findings established the specificity of the test system.

The concentrated organisms, after centrifugation, were resuspended in a minimal amount of distilled water. In one experiment with *M. hominis* type 2, when the concentrate of organisms was suspended in physiological saline

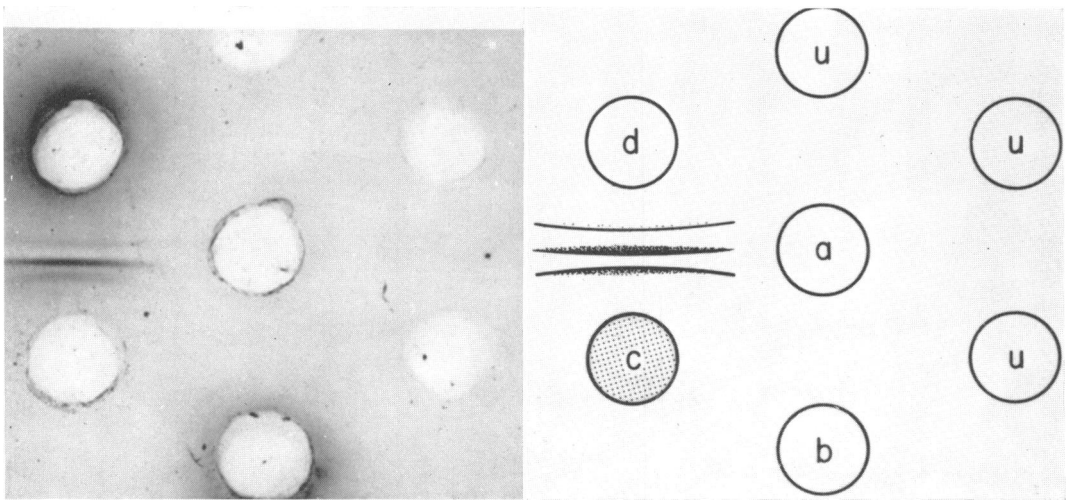

FIG. 1. *Demonstration of specificity of reaction in gel diffusion. The precipitation bands were stained with azocarmine B. In the drawing, the reservoir with antigen is shadowed. Symbols: a = deposit from centrifugation of uninoculated rabbit infusion broth; b = preimmunization rabbit serum; c = Mycoplasma hominis type 2 antigen; d = M. hominis type 2 hyperimmune rabbit serum; u = unfilled cups.*

the material was not antigenic. Freezing and thawing of the latter preparation six times did not result in line formation, not did it apparently increase the antigenic potency of the material suspended in distilled water. We did not evaluate the effect of freezing and thawing on all our preparations, however, and continued to use this step in the production of antigen.

Antigenic relationships of M. hominis type 1. To study the relationships between the established strain, *M. hominis* type 1, and isolates DC63, V2785, and Botte, four patterns were arranged so that each of the antigens in a central cup was tested against the four different antisera in the peripheral wells. The *M. hominis* type 1 preparation formed a single band with its antiserum. This band fused with a single precipitation line formed by the *M. hominis* type 1 antigen and antisera for the three oral isolates. Antigen preparations of the oral isolates revealed a more complex pattern, in which three precipitation bands formed with the homologous antiserum; these lines fused with a similar set of three lines which developed with the heterologous antisera for the oral strains and *M. hominis* type 1. A representative pattern for the V2785 antigen preparation is shown in Fig. 2. When antiserum for the oral isolates or *M. hominis* type 1 was placed in the central cup, three bands developed with the oral isolate antigens, and one band formed with the *M. hominis* type 1 antigen. The three bands were continuous and at least one of them fused with the precipitation line produced by the *M. hominis* type 1 antigen. These findings suggested that the three oral isolates were identical and closely related to *M. hominis* type 1.

A more detailed analysis of the relation of *M. hominis* type 1 to the oral isolates is shown in Fig. 3. At least two of the precipitation bands between DC63 antigen and antisera to DC63 and *M. hominis* type 1 fused with lines between *M. hominis* type 1 antigen and the same antisera. A similar pattern was observed when V2785 and *M. hominis* type 1 antigens were compared. However, one of the lines formed by DC63 and V2785 antigens was absent when *M. hominis* type 1 antigen was used. This finding suggests that *M. hominis* type 1 is deficient in an antigen possessed by the oral isolates. A small quantity of this component is present in *M. hominis* type 1, however, since antiserum against the organism contains antibody reactive with each of the antigenic components of DC63 or V2785. From this we concluded that the three oral isolates, DC63, V2785, and Botte, were identical and very closely related to *M. hominis* type 1. These relationships were confirmed, using antigens and antisera prepared with guinea pig materials.

Antigenic relationships of M. hominis type 2.

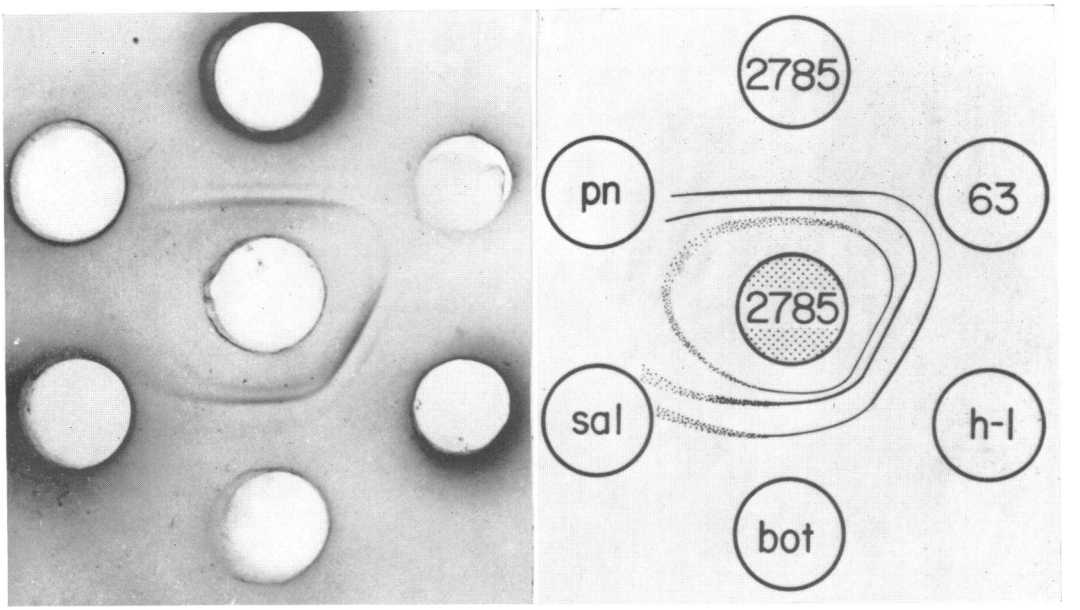

FIG. 2. *Relation between Mycoplasma hominis type 1 and human oral isolates DC63, V2785, and Botte. The bands were stained with azocarmine B. The antigen was prepared from organisms grown in rabbit infusion; rabbit antisera were used. Contents of the reservoirs, starting at the center and proceeding clockwise around the periphery: V2785 antigen; V2785 antiserum; DC63 antiserum; M. hominis type 1 antiserum; Botte antiserum; M. salivarium antiserum; M. pneumoniae antiserum.*

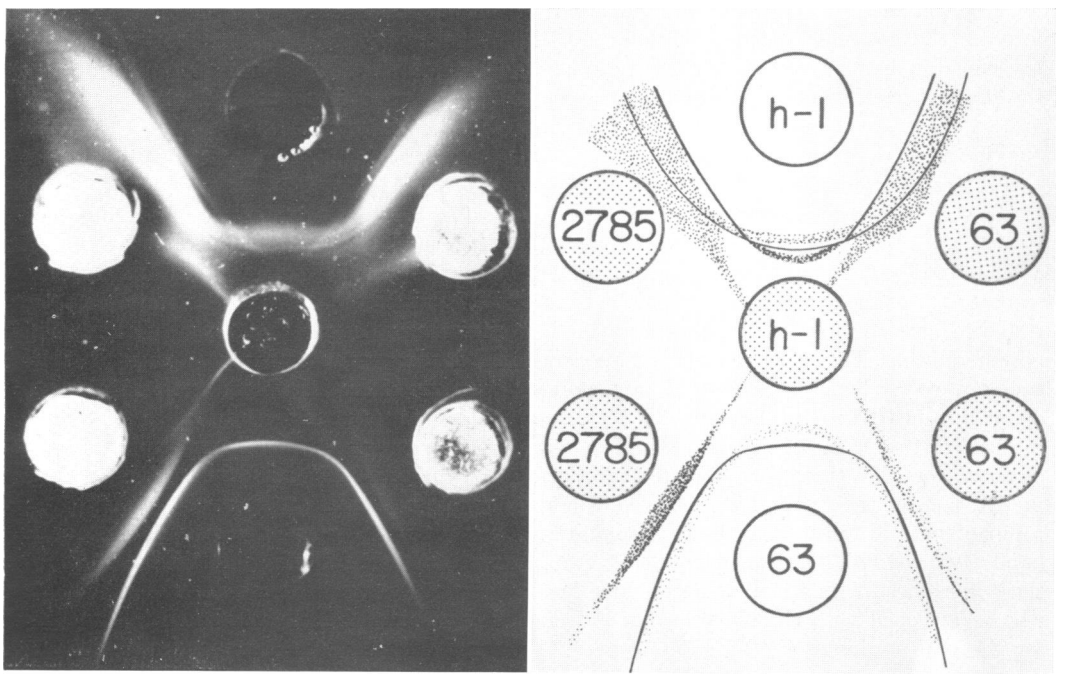

FIG. 3. *Antigenic difference between Mycoplasma hominis type 1 and the two isolates DC63 and V2785. The bands were not stained. The antigens (denoted by shading) were prepared from organisms grown in rabbit infusion broth; all antisera were obtained from rabbits. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: M. hominis type 1 antigen; M. hominis type 1 antiserum; DC63 antigen; DC63 antigen; DC63 antiserum; V2785 antigen; V2785 antigen.*

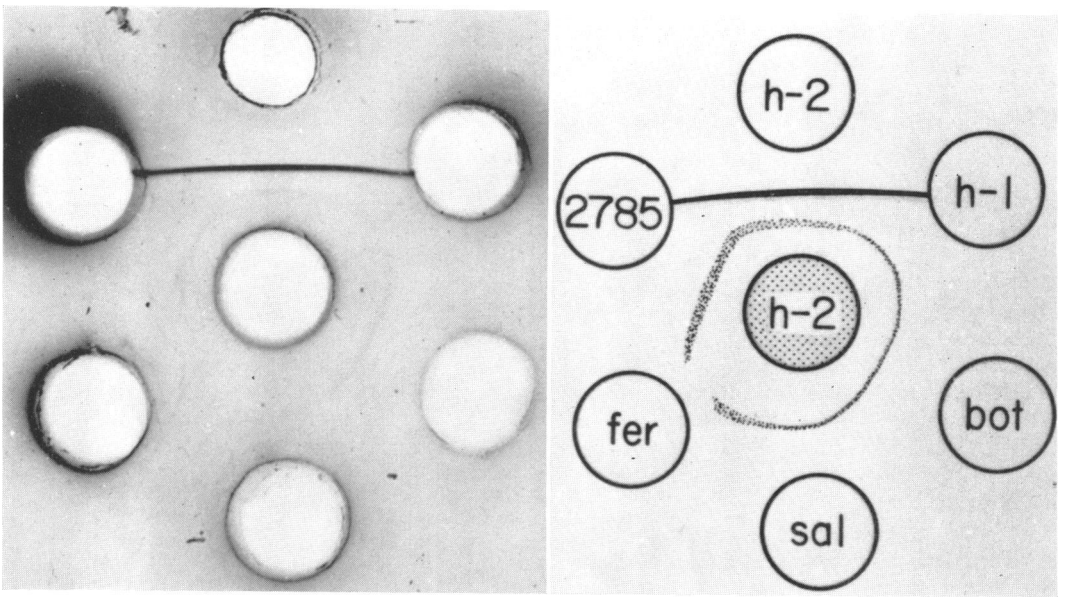

FIG. 4. *Specificity of antigenic components of Mycoplasma hominis type 2. The bands were stained with azocarmine B. The antigen was prepared from organisms grown in rabbit infusion broth; all antisera were obtained from rabbits. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: M. hominis type 2 antigen; M. hominis type 2 antiserum; M. homonis type 1 antiserum; Botte antiserum; M. salivarium antiserum; M. fermentans antiserum; V2785 antiserum.*

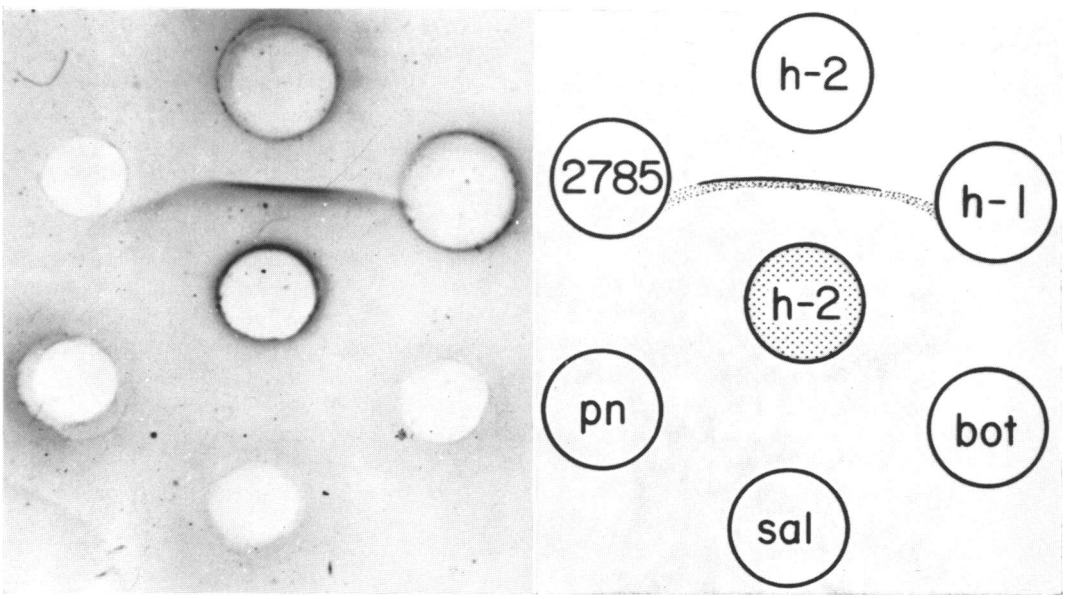

FIG. 5. *Specificity of Mycoplasma hominis type 2 confirmed with the guinea pig system. The bands were stained with azocarmine B. The antigen was prepared from organisms grown in a guinea pig infusion broth; all antisera were obtained from guinea pigs. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: M. hominis type 2 antigen; M. hominis type 2 antiserum; M. hominis type 1 antiserum; Botte antiserum; M. salivarium antiserum; M. pneumoniae antiserum; V2785 antiserum.*

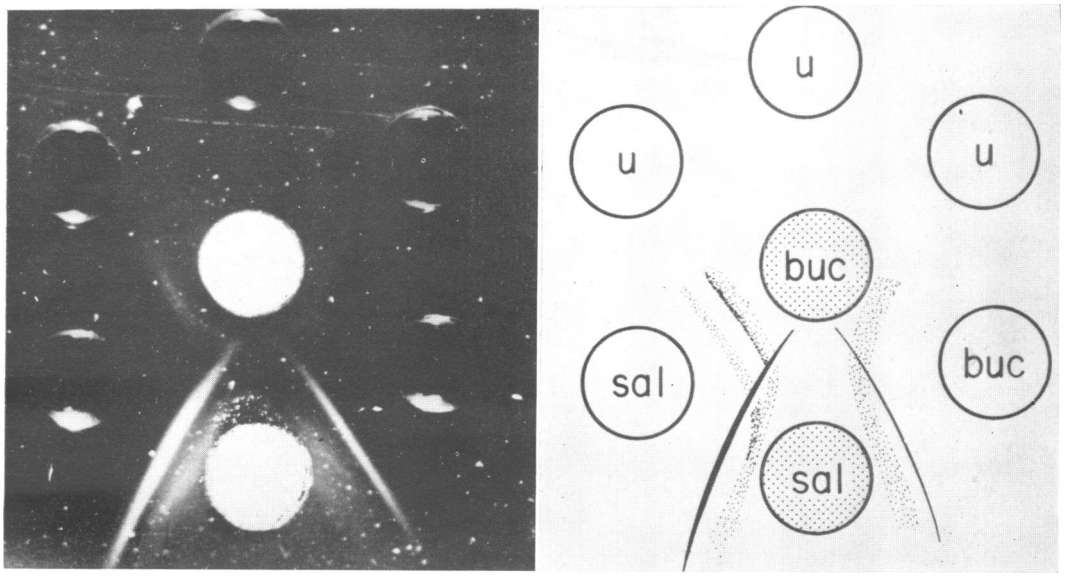

FIG. 6. *Relation of Mycoplasma salivarium to the Buccal strain. The bands were not stained. The antigens (denoted by shadowing) were prepared from organisms grown in rabbit infusion broth; all antisera were obtained from rabbits. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: Buccal antigen; two unfilled cups (denoted by "u"); Buccal antiserum; M. salivarium antigen; M. salivarium antiserum; unfilled cup.*

Gel diffusion studies on *M. hominis* type 2 (genital strain Campo, PG27) revealed at least one antigenic component shared with all the other mycoplasmas; in some diffusion patterns we observed as many as three antigenic components in common with other strains (DC63, Buccal, and DC585), depending upon the heterologous antisera used. In addition, the Campo strain appeared to contain one or two components which are not shared with other organisms. This specificity was demonstrated in both rabbit and guinea pig systems (Fig. 4 and 5).

Antigenic relationships of M. salivarium. Since the Buccal and DC585 oral isolates had shown complete cross-reactivity with *M. salivarium* PG20 in the complement-fixation test, we investigated the relationships of these strains by gel diffusion. When tested in a homologous system, *M. salivarium* contained at least two antigenic components. The *M. salivarium* and Buccal antigens and antisera were tested as shown in Fig. 6. Antigenic materials from these two strains showed cross reactions. However, they appeared to be distinct, since an antigenic component of *M. salivarium* was not detectable in the Buccal strain. Probably the Buccal strain contains a small amount of this component, since antiserum against the organism contains antibodies reactive with each of the antigens of *M. salivarium*.

In other tests, strain DC585 was identical with the Buccal strain. Apart from the interrelationships mentioned, we were able to show that *M. salivarium* PG20, Buccal, and DC585 strains had antigens in common with other mycoplasmas. We placed six different antigens, including *M. salivarium*, in circumferential wells and added *M. salivarium* antiserum to the central reservoir. At least one precipitation line was common to all the strains tested (Fig. 7), but there was, in addition, a specific band between *M. salivarium* antigen and its antiserum. We extended these observations in other gel diffusion patterns and also showed that the Buccal and DC585 strains possessed at least one line in common with other mycoplasmas. Additional relationships involving *M. salivarium* are illustrated in Fig. 8

Antigenic relationships of M. pneumoniae. M. pneumoniae could not be grown in a rabbit or guinea pig infusion broth. Therefore, we grew this organism in complete PPLO broth. An antigen prepared from a 6-day-old culture gave a weak precipitation band with hyperimmune rabbit serum. This precipitate was not observed

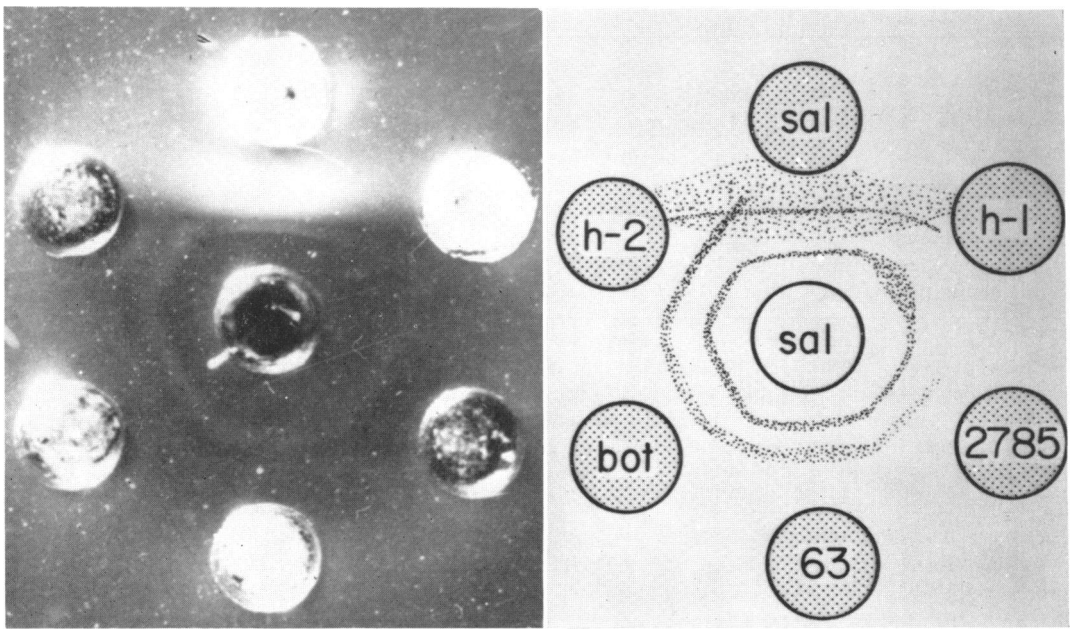

FIG. 7. *Specificity of antigenic components of Mycoplasma salivarium. The bands were not stained. All antigens were prepared from organisms grown in rabbit infusion broth; the antiserum was produced in a rabbit. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: M. salivarium antiserum; M. salivarium antigen; M. hominis type 1 antigen; V2785 antigen; DC63 antigen; Botte antigen; M. hominis type 2 antigen.*

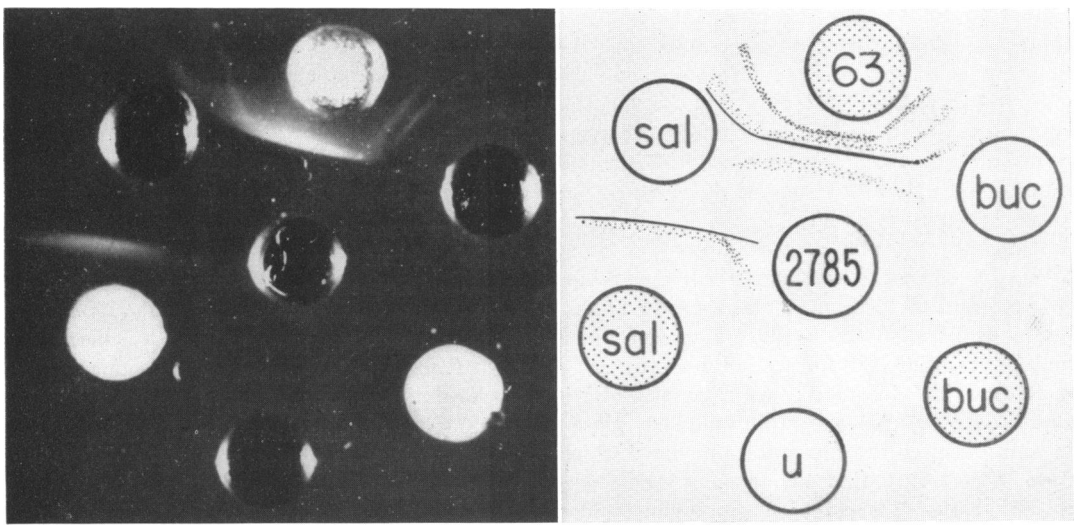

FIG. 8. *Common antigens between Mycoplasma salivarium and oral isolates, which are related to M. hominis type 1. The bands were not stained. The antigens (denoted by shadowing) were prepared from organisms grown in rabbit infusion broth; the antisera were obtained from rabbits. Contents of the reservoirs, starting at the center well and proceeding clockwise around the periphery: V2785 antiserum; DC63 antigen; Buccal antiserum; Buccal antigen; unfilled reservoir; M. salivarium antigen; M. salivarium antiserum. The reaction generally observed between Buccal antigen and antiserum was not present at the time of photography.*

TABLE 3. *Relation of oral isolates to Mycoplasma hominis type 1 by immunofluorescence*

Rabbit serum	Intensity of fluorescence with indicated colonies and a 1:40 dilution of serum*							
	M. hominis				*M. hominis* type 2	*M. salivarium* PG20	*M. fermentans* G	*M. pneumoniae*
	Type 1	DC63	2785	Botte				
DC63 preimmunization	0	0	0	0	0	0	0	0
DC63 postimmunization	0	3–4	2–3	1–2	0	0	0	0
Homologous preimmunization	0	0	0	0	0	0	0	0†
Homologous postimmunization	3–4	3–4	1–2	1–2	3–4	4	1–2	3–4†

* *Mycoplasma* colonies were transferred from agar plates to glass slides and then tested by the indirect-immunofluorescent technique, using either antirabbit or antihuman globulin conjugated with fluorescein isothiocyanate. Symbols: 4 represents the most intensive fluorescence; 3 to 2 represents intermediate intensity of fluorescence; 1 signifies the least amount of fluorescence; 0 indicates no fluorescence.

† Paired sera from a patient with *M. pneumoniae* infection (pneumonia) used at a 1:10 dilution.

before 48 hr of incubation in the gel diffusion system. The preimmunization serum did not react, nor did a preparation from uninoculated broth medium, a result that indicated the specificity of the system. Materials prepared from cultures that had been incubated for periods longer than 6 days did not produce a line of precipitate. We also obtained an antigen by scraping the confluent surface growth from agar plates. This preparation also produced a weak band of precipitation which fused with the line formed by the antigen prepared in the complete broth. A definite line was shown only with the homologous serum. A very faint, diffuse line was observed with *M. hominis* type 2 serum, but this reaction was not considered definite. Antiserum prepared against *M. pneumoniae* gave one precipitation band when tested against *M. hominis* type 1 and *M. salivarium* (Fig. 2).

Antigenic relationship of a simian 698 strain to human strains. To explore the relationships between isolates of human and those of animal origin, we studied the simian 698 strain which had been isolated from the oropharynx of a monkey. In an initial study with the 698 antigen against six heterologous rabbit antisera placed in outer cups, the precipitation lines which formed indicated that the simian strain was related to the *M. hominis* type 1 organism and those isolates closely related to type 1. This finding was confirmed with the guinea pig system. However, when the simian antigen and DC63 antigen were placed in proximity to one another and tested against the simian antiserum, an additional precipitation line was demonstrated with the homologous system.

Immunofluorescence studies. In gel diffusion studies, oral strains DC63, V2785, and Botte were indistinguishable. However, they could be differentiated from *M. hominis* type 1. In complement-fixation tests all four strains were alike. Using immunofluorescence, the oral strains and the recognized strains *M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, and *M. pneumoniae* were tested against preimmunization and hyperimmune DC63 rabbit antiserum. The results (Table 3) confirm the difference between the three oral strains and the established *M. hominis* type 1 strain. Further, in this test there was no relationship evident between these strains and the other four recognized strains.

DISCUSSION

Edward and Freundt (1956) suggested a classification of mycoplasma in which biological and biochemical properties are used for characterization of species. The four human species recognized by Edward and Freundt (1956), *M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, and *M. fermentans*, can also be distinguished by the agglutination or complement-fixation technique (Nicol and Edward, 1953; Card, 1959; Coriell, Fabrizio, and Wilson, 1960). In several instances, however, extensive cross reactions between species have been observed in complement-fixation tests (Card, 1959; Coriell et al., 1960). In addition, Villemot and Provost (1959), using gel diffusion techniques, described antigens which are shared by human, bovine, avian, and saprophytic mycoplasmas.

The significance of these cross reactions, however, is uncertain, since growth medium in-

gredients, which might in themselves be antigenic, were common to the immunizing material and the antigens used in complement-fixation and gel diffusion tests. In each instance, a beef infusion or yeast extract component was common to the immunizing and test antigens.

It is difficult to remove contaminating growth constituents from suspensions of organisms used for immunization. Most mycoplasmas require a complex growth medium, which usually contains a meat infusion to which serum or ascitic fluid is added. It was not possible to grow the human strains of mycoplasma in a synthetic medium; therefore, we used a medium which was not foreign to the animal inoculated in the immunization procedure. We grew the mycoplasma in a rabbit or guinea pig infusion medium, with corresponding rabbit or guinea pig serum. Under these conditions, immunized animals should develop antibody for mycoplasma antigens, but not for growth medium components. Because of the manner in which immune sera were prepared, it is probable that the low-level heterologous reactions represent a true sharing of antigenic constituents. The need for preparing antisera in a homologous system would appear to be of even greater importance in gel diffusion studies; apart from *M. pneumoniae*, the antigens employed in double-diffusion tests were the same as those used in the immunization procedure.

All antigen preparations did not have the same potential for producing complement fixation or lines of precipitate in double diffusion. The antigen preparations for gel diffusion were not standardized, since the amount of growth obtained varied with different strains, and cultures were incubated longer than the point of maximal viability. Despite this fact, we could observe precipitation lines within 2 hr after addition of most antigens and antisera to the reservoirs. However, with two antigens, *M. salivarium* and *M. pneumoniae*, the precipitation bands appeared much later. Another preparation of *M. salivarium*, however, produced lines within 2 hr; this rapid reaction was never observed with the *M. pneumoniae* strain. The fact that *M. pneumoniae* grew relatively poorly on our medium probably explains our difficulty in preparing a satisfactory antigen (Chanock et al., 1962*a*). The method used for disrupting organisms was found to be important in preparing satisfactory antigens for agar gel diffusion. To accomplish this, we used osmotic shock with distilled water, but it is possible that other methods of producing lysis, such as treatment with surface-active agents or solvents, may give better results.

The results of our complement-fixation tests and gel diffusion studies (Table 4) show good correlation with the classification of human "prototype" strains proposed by Edward and Freundt (1956). Further, we were able to identify our unclassified isolates with some of these prototype strains. Human oral strains DC63, V2785, and Botte appeared to be identical or very closely related to the genital strain, *M. hominis* type 1. Certainly, by complement-fixation these four strains of mycoplasmas appeared to be the same. However, our evidence indicated that the gel diffusion technique was more specific and that *M. hominis* type 1 was deficient in an antigen present in the three oral strains. This observation was substantiated by immunofluorescence studies. Similarly, in gel diffusion, DC585 and Buccal strains seemed to be closely related to, but distinguishable from, *M. salivarium*. Thus, in gel diffusion studies we were able to show cross relationships among the mycoplasmas, and at the same time detect specific components. Even strains which appeared to be specific antigenically by complement-fixation tests were shown by gel diffusion to share antigens with other mycoplasmas. The cross relationships among human mycoplasmas shown by other workers can be explained, at least partially, on the basis of the sharing of a common antigen or antigens.

The results of both the complement-fixation and gel diffusion studies suggest that *M. pneumoniae* is antigenically distinct from other human mycoplasmas. However, the antigenic preparation for gel diffusion, obtained from *M. pneumoniae* is inferior to materials produced with other mycoplasmas. Since the antiserum to *M. pneumoniae*, which had a high homologous complement-fixation titer, produced a weak precipitation band when tested against other mycoplasma, a more potent preparation of *M. pneumoniae* might produce evidence of other cross relationships. It was surprising that the only antigenic preparation of *M. pneumoniae* which was active in gel diffusion was prepared from a 6-day-old culture, whereas the maximal yield of organisms occurred at 9 to 10 days (Chanock et al., 1962*a*). Although we have no satisfactory explanation for this phenomenon, we can speculate that the antigenic component measured in

TABLE 4. *Relation of mycoplasma and recent human oral isolates by complement-fixation and agar double diffusion*

Mycoplasma	Relation of antigen to other mycoplasmas in indicated tests	
	Complement-fixation	Gel diffusion
M. hominis type 1		Closely related to DC63, V2785, and Botte, but lacks specific band; shares at least one antigen with *M. hominis* type 2, *M. salivarium*, and *M. pneumoniae*
M. hominis isolates DC263, V2785, Botte	Indistinguishable	Indistinguishable; shares at least one antigen with *M. hominis* type 2 and *M. salivarium*
M. hominis type 2 Campo, PG 27	Specific	Two specific bands; shares at least one other antigen with *M. hominis* type 1 and *M. salivarium*
M. salivarium PG20, Buccal	Indistinguishable	One specific and one shared band; also shares one antigen with *M. hominis* types 1 and 2 and *M. pneumoniae*
Isolate DC585		Identical to Buccal
M. pneumoniae	Specific	Specific band; probably shares at least one band with *M. hominis* type 2
M. fermentans	Specific	Not done

gel diffusion is soluble and diffuses into the growth medium.

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