Characteristics of Virulent, Attenuated, and Avirulent *Mycoplasma pneumoniae* Strains

RICHARD P. LIPMAN, WALLACE A. CLYDE, JR., AND FLOYD W. DENNY

Departments of Pediatrics and Bacteriology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Homologous pairs of virulent and attenuated or avirulent *Mycoplasma pneumoniae* strains were derived and compared in an effort to elucidate the mechanisms of virulence. These related strains were found to vary in growth, glycolysis, protein electrophoretic patterns, peroxide formation, morphology, and cytadsorption. Variations in the last two characteristics closely correlated with avirulence. This enables understanding of one stage in the pathogenic sequence and provides a convenient marker for avirulence. The derivation of infectious avirulent strains may make possible their use as live vaccines against *M. pneumoniae* disease.

Mycoplasma pneumoniae is an important cause of human lower respiratory infection, but little is known of the mechanism by which it produces disease. Until recently, organism characteristics such as cytadsorption (20) and peroxide formation (21), thought to mediate pathogenicity by direct damage to adsorbing tracheal epithelium, have been studied in *M. pneumoniae* strains of undefined virulence. The present investigation was initiated to examine several characteristics of strains whose virulence had been determined first by controlled in vivo studies. In experiments reported previously (14), it proved possible to derive two pairs of homologous virulent and attenuated strains from two parent strains of *M. pneumoniae* by repeated passage in artificial media or in a susceptible host. Pathogenicity of the strains was defined and quantitated in the hamster model. The study further described variations in peroxide formation and adsorption to erythrocytes; cytadsorption appeared to be required for virulence. These differences suggested that other characteristics might vary among the strains and perhaps be related to pathogenicity.

The present report extends and supplements the previous work. A variety of techniques was utilized to study morphology, growth, selected metabolic properties, protein electrophoresis, and antigenic cross-reactions. Also described is a new avirulent strain produced by growth in a semidefined medium.

MATERIALS AND METHODS

Media. Cultivation utilized Hayflick's medium (2) containing 70% PPLO Broth or Agar (Difco), 20% unheated horse serum, and 20% aqueous yeast extract (Robbin Laboratories). In an attempt to avoid stimulation of antibodies to the growth medium, antigens for the production of antisera in rabbits were grown in 67.5% Medium 199, 20% pooled rabbit serum heated at 56 C for 30 min, 10% diffusate of pressure-dialyzed yeast extract, and 2.5% (v/v) of a 7.5% sodium bicarbonate solution (Abbott; 10). The growth medium referred to as "semi-defined" consisted of 94.5% Medium 199, 2.5% (v/v) sodium bicarbonate solution, 2% PPLO Serum Fraction (Difco), and 1% diffusate of dialyzed yeast extract. All media contained penicillin G (1,000 units/ml), and liquid media contained 1% glucose and 0.004% phenol red indicator. Cultures in media containing Medium 199 were maintained in a 5% CO_2 atmosphere to stabilize their initial pH of 7.2.

Organisms. Homologous virulent and attenuated pairs based on pathogenicity testing in hamsters were derived from parent strains of *M. pneumoniae* as described previously (14). Strain Mac-H106, fully virulent, resulted from eight successive passages in hamsters and four broth passages of the attenuated parent strain, Mac-K (Table 1). A second parent strain, M129-B6, was virulent; it gave rise to the avirulent strain, M129-B169, the 169th broth passage. Another M129-B6 derivative, not described previously, was the result of eight passages in semi-defined medium; this strain, M129-M8, infected all hamsters inoculated intranasally but produced no histological pneumonia, and thus was avirulent. The identity of all strains as *M. pneumoniae* was proven by inhibition of growth on agar by specific antiserum discs (5). A single pool of each organism, stored in portions at −65 C, was utilized for all experiments.

Electron microscopy. Samples from single broth cultures during late log phase and late stationary growth phase were concentrated 10-fold after washing in 0.85% NaCl with 0.02 M phosphate buffer at pH 7.2 (PBS). Droplets of the suspension were placed on

agar squares, fixed in 40% Formalin vapor at 4 C, and shadowed as described by Kim et al. (13).

Growth curves. Growth in stationary broth cultures at 37 C was measured daily by inoculating serial 10-fold dilutions onto PPLO agar plates (13). The colony-forming units (CFU) were counted on the 10th day.

Polyacrylamide gel electrophoresis. A modification of the techniques of Razin and Rottem (17) and Takayama et al. (24) was employed. The gel, containing 7.5% acrylamide, 5 M urea, and 35% acetic acid was prepared by combining three solutions. Solution A contained acrylamide (Eastman Chemical Products, Inc., Kingsport, Tenn.), 10 g; urea (Baker), 20 g; N,N'-methylenebisacrylamide (Eastman), 0.27 g; glacial acetic acid, 47 ml; and glass-distilled water (GDW) to make a total volume of 100 ml. Solution B contained urea, 60 g; N,N,N',N'-tetramethyl-ethylenediamine (Eastman), 2 ml; and GDW to make 100 ml. Solution C, prepared fresh, contained 1.5 g of ammonium persulfate (Fisher Scientific Co., Pittsburgh, Pa.), and GDW to make 10 ml. To form the gel, 3 volumes of solution A, 1 volume of solution B, and 0.1 volume of solution C were combined in that order and mixed gently; 3 ml was added to glass columns (100 by 8 mm) sealed at the lower end with plastic sheeting. The solution was overlaid with 0.5 ml of 75% acetic acid and underwent polymerization in darkness at 37 C for 1 hr. Specimens of each strain grown in broth were processed as described by Razin and Rottem (17). Prior to treatment with phenol-acetic acid, all strains were adjusted to equivalent concentrations of protein (15). After polymerization, gel columns were overlaid with a mixture of 0.1 ml of 40% sucrose in 35% acetic acid and 0.2 ml of specimen, which contained approximately 0.2 mg of protein. Successive overlays of 0.5 ml of 75% and 10% acetic acid were then placed. Electrophoresis proceeded for 1 hr at 2 ma per column to reduce convection mixing and then for 3 hr at 5 ma per column. After overnight staining with 1% amido black, the columns were destained electrolytically in oversized glass tubes plugged with gel.

Antisera. Cultures of each strain grown in rabbit serum medium were harvested by centrifugation for 1 hr at 15,000 × g when the pH had fallen to 6.8. M129-M8 was harvested when its semidefined medium became turbid. The antigens were washed once, resuspended in PBS to a protein content of 0.5 mg/ml, and stored at −65 C until used. Albino male rabbits were immunized intravenously every other day with 0.5 ml of antigen for a total of five doses. Two days after the final injection, a mixture of 0.5 ml of antigen and 0.5 ml of Freund's Complete Adjuvant (Difco) was administered intramuscularly. Serum was obtained prior to the injections and at 14, 28, and 56 days after the first injection. All sera were heated for 30 min at 56 C prior to use.

Serological studies. The complement fixation (CF) test, by the method of Sever (18), was performed by microtitration. Antigen was prepared by the method of Kenny and Grayston (12). Two units of antigen, standardized against homologous antiserum in a checkerboard titration, were reacted with twofold dilutions of homologous and heterologous antisera and 2 units of complement with overnight fixation at 4 C.

The metabolic inhibition (MI) test was performed by microtitration by the technique of Fernald et al. (10).

Double immunodiffusion (ID) was performed with lucite templates (Matrix for micro immunodiffusion no. 17400-9300, Mann) set upon a 1 mm-thick layer of 1% Special Noble Agar (Difco) and 0.05% sodium azide in PBS on glass microscope slides (25 by 75 by 1.2 mm). Antigens were prepared by concentrating 500-fold in PBS thrice-washed broth cultures of each strain. The concentrates underwent 20 freeze-thaw cycles, and their protein content was adjusted to approximately 70 mg/ml. Adjacent template wells were filled with serum and antigens, and double diffusion proceeded for 5 days at room temperature in a humidified atmosphere. After removal of the templates, the slides were washed, dried, stained in 1% amido black, and rinsed as described in the LKB 6800A instruction manual.

Peroxide formation. Peroxide formation by M129-M8 was evaluated qualitatively by its ability to produce hemolysis. Colonies grown on PPLO agar for 10 days were overlaid with 3% sheep erythrocytes and 1% Bacto-agar (Difco) in PBS and observed every 12 hr for hemolysis (4).

Cytadsorption. The cytadsorptive ability of M129-M8 was measured by the technique of Sobeslavsky et al. (20). Colonies grown on PPLO agar for 6 days were overlaid with 0.2% chicken erythrocytes in 0.85% NaCl and observed for residual hemadsorption after incubation and washing.

RESULTS

A summary of the derivation and pathogenicity of the strain pairs is presented in Table 1. Two strains, M129-B169 and M129-M8, produced no pneumonia and thus were avirulent; strain Mac-K was attenuated, producing pneumonia in four of nine hamsters. Strains M129-B6 and Mac-H106 were fully virulent. A variety of techniques was used to compare the parent strains with their respective derivatives.

Morphology. By light microscopy the colony morphology of all strains grown on PPLO agar was identical. However, two distinct morphological patterns in broth could be discerned. All strains formed smooth, refractile spherular clusters up to 400 μm in diameter (Figure 1A) except M129-B169 and M129-M8. Clusters of these two avirulent strains were smaller, amorphous, granular, and nonrefractile, as in Fig. 1B. By electron microscopy, all strains progressed similarly through two morphological states, apparently correlated with growth phase. During the log phase all strains (except M129-M8, which yielded no satisfactory preparations) formed only spheres; these occurred singly or in clumps and ranged in size from 200 to 700 nm (Fig. 2A).

TABLE 1. *Derivation, infectivity, and pathogenicity of M. pneumoniae strains*

Strain	Passage history[a]	Infection (hamsters)		Pneumonia (hamsters)	
		Positive/no. inoculated	Mean CFU[b]	Positive/no. inoculated	Mean severity[c]
MAC					
K (parent)	E78, A21, B123	9/9	5.78	4/9	1.0
H106 (derivative)	E78, A21, B123, H8, B4	12/12	5.81	12/12	2.3
M129					
B6 (parent)	B6	6/6	5.38	6/6	2.3
B169 (derivative)	B169	9/9	5.51	0/9	
M8 (derivative)	B6, M8	15/15	5.59	0/15	

[a] E, chick embryo; A, agar; B, broth; H, hamster; M, semidefined medium.
[b] Expressed as mean CFU per gram of lung ($\log_{10}$).
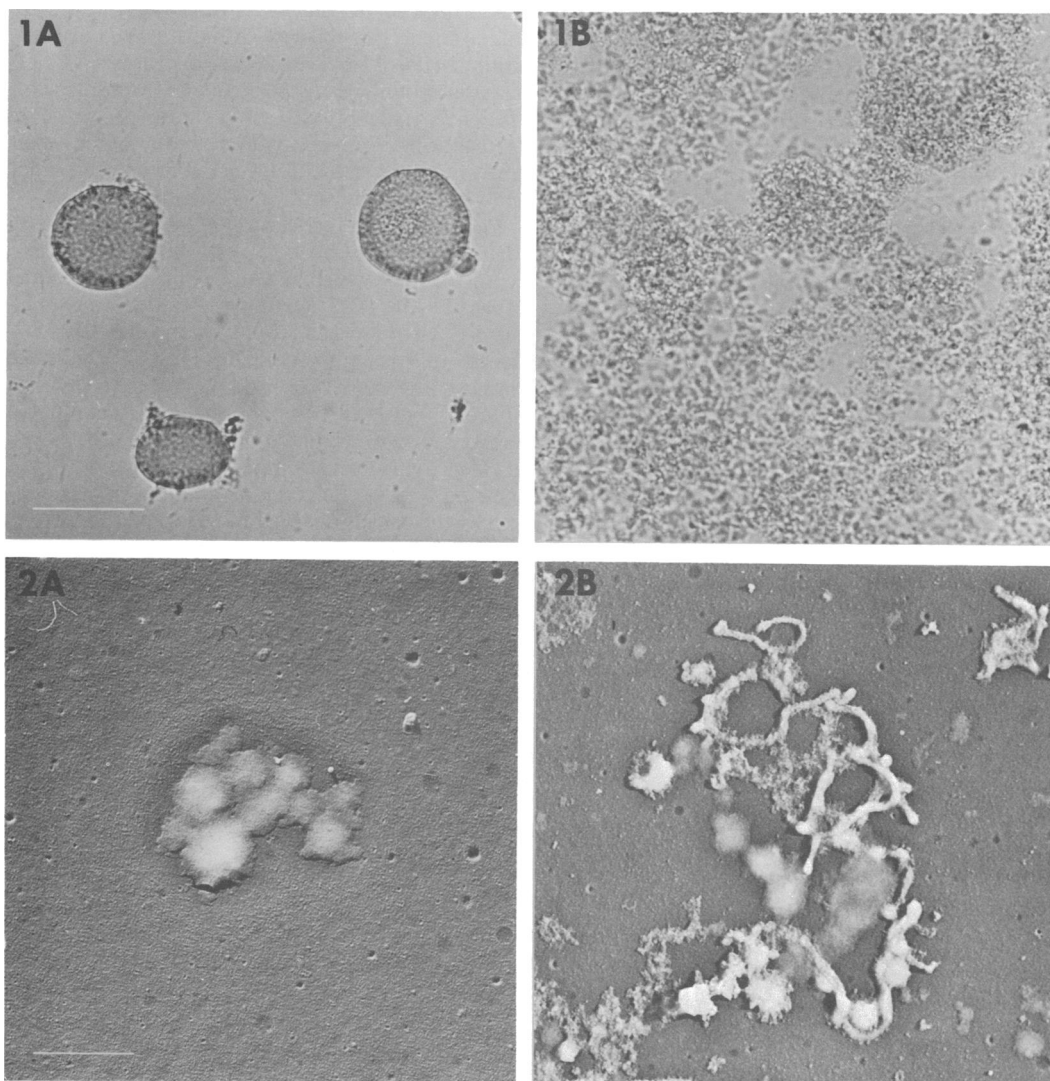[c] Histopathological extensiveness in pneumonia-positive animals on a scale of 1 to 4.

FIG. 1. *Representative morphology of M. pneumoniae strains grown in broth. (A) Spherular colonies formed by attenuated strain Mac-K and virulent strains Mac-H106 and M129-B6. (B) Granular amorphous colonies formed by avirulent strains M129-B169 and M129-M8. Bar 400 μm.*

FIG. 2. *Representative electron photomicrographs showing typical morphology of virulent and avirulent strains at two phases of growth. (A) Spherical form of all strains during late log phase. (B) Filamentous forms of all strains during stationary phase. Bar 1 μm.*

Their surface detail was very indistinct, as if obscured with amorphous material. During stationary phase, the spheres became flattened and more distinct, extruding multiple filaments and buds, as in Fig. 2B. Thus, distinctive strain-related differences were seen only by light microscopy of broth cultures.

Growth. All strains were studied by comparison of growth curve and generation time (GT). Three strains (Mac-K, Mac-H106, and M129-B6 had virtually identical growth curves (Fig. 3), with a GT of approximately 10 hr. The GT of the two avirulent M129 strains differed markedly from the virulent parent; with M129-B169 it was shortened (4.6 hr), and with M129-M8 it was prolonged (16.5 hr). The higher numbers of CFU of M129-B169 presumably were related to greater dispersion of reproductive units, since its maximum protein yield was almost identical to that of M129-B6. In contrast, a medium possibly deficient in nutrients may have retarded the growth of M129-M8, whose protein mass per unit of medium was only one-third that of the other strains. Strain variations, as demonstrated by these differences in growth, were unrelated to virulence.

Glycolysis and peroxide formation. The ability of the virulent, attenuated, and avirulent strains to ferment glucose in broth, with a resultant fall in *p*H, was studied qualitatively. All strains fermented glucose except M129-M8, which in its semidefined medium produced no acidity during any stage of growth. The lack of glycolysis with M129-M8 might have resulted from impaired metabolism in a nutritionally deficient medium; however, the variation persisted for five subsequent passages in Hayflick's broth, suggesting an

actual alteration in the organism. Peroxide formation was studied by a simple quantitative method reported in detail elsewhere (14). It was found that, although there are definite quantitative differences in peroxide formation between strains, homologous attenuated and virulent pairs produce equal amounts of peroxide. This observation was extended by the finding that M129-M8, an avirulent strain, produced amounts of peroxide equivalent to those of its virulent parent.

Cytadsorption. Extension of previous work comparing the cytadsorbing ability of *M. pneumoniae* strains (14) was conducted utilizing M129-M8. Erythrocytes did not adsorb to colonies of this strain, as was the case with the other avirulent strain, M129-B169. Thus, in two strains a loss of hemadsorption was associated with a total loss of virulence, suggesting that cytadsorption is a requisite for virulence. Nevertheless, other virulence factors must also be necessary since strain Mac-K cytadsorbs and yet is attenuated.

Polyacrylamide gel electrophoresis. Twenty-five identical bands were obtained for all strains except M129-M8 which had nine (Fig. 4). The bands of M129-M8 (Fig. 4E) corresponded to bands formed by the other strains, but some were reduced in protein content, as judged by their staining intensity. Thus, no differences in electrophoretic patterns between the strains could be associated with variations in virulence. Attempts at identifying bands containing antigens by immersing washed gel columns in antisera were unsuccessful, as was an attempt to locate lipoprotein by staining with Sudan Black B, suggesting denaturation of the proteins by the phenol-acetic acid treatment.
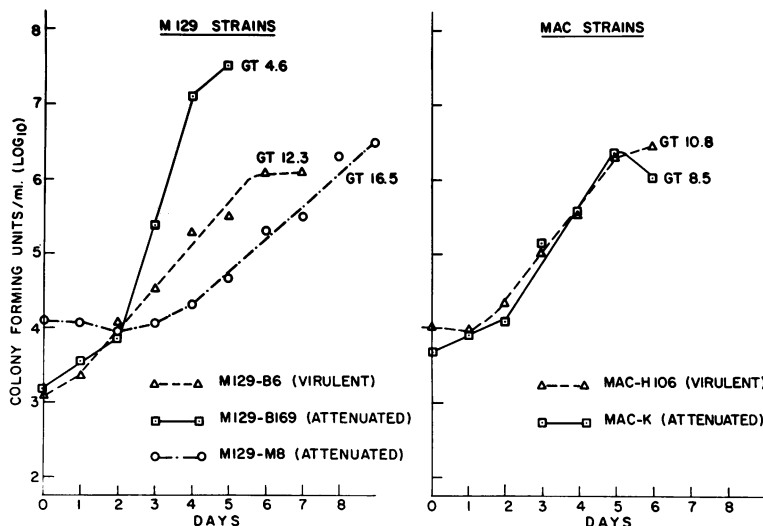

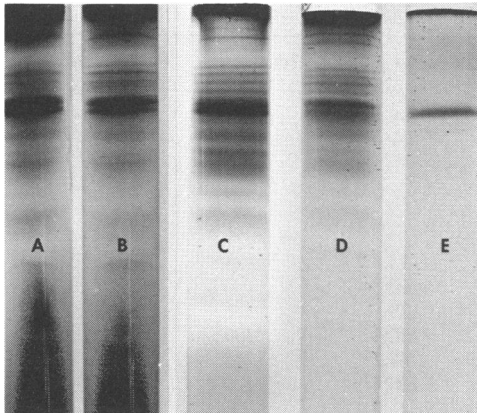FIG. 3. *Growth curves and GT of virulent and attenuated or avirulent M. pneumoniae strains in broth.*

FIG. 4. *Protein composition of M. pneumoniae strains compared by polyacrylamide gel electrophoresis. (A) Mac-K, (B) Mac-H106, (C) M129-B169, (D) M129-B6, and (E) M129-M8.*

In control preparations, simultaneous electrophoresis of Hayflick's broth yielded 16 bands, only 5 of which occupied the same positions as bands formed by the *M. pneumoniae* strains. These five broth bands originated entirely from the horse serum component and were also present with PPLO serum fraction. The fact that the other 11 broth bands, many of which contained large amounts of protein, were entirely eliminated from the organism preparations indicated the adequacy of the washing procedure. These observations also suggest, however, that *M. pneumoniae* selectively incorporates several proteins from the growth medium. Whether these substances actually represent structural components characteristic of *M. pneumoniae* will not be known until comparative study is enabled by development of additional growth media.

Antigenic comparisons. Antigens and antisera representing homologous virulent and attenuated strain pairs were studied for cross-reactivity in the CF, MI, and ID tests. The CF and MI titration results are presented in Tables 2 and 3. Also shown is the relatedness between strain pairs as determined from the formula applied by Archetti and Horsfall (1), in which a value of 100% indicates complete cross-reactivity. The titers and relatedness values demonstrate complete antigenic identity among the strain pairs. Since strain M129-M8 does not ferment glucose, the MI reaction could not be compared in this instance. The ID test, in which a single antiserum was allowed to react simultaneously with four homologous and heterologous antigens, showed up to eight lines of apparent identity between all combinations of antisera and antigens. A representative preparation is shown in Fig. 5. In additional ID tests,

human convalescent serum obtained 2, 3, and 4 weeks after natural infection reacted identically with antigens from virulent and avirulent strains, further indicating a lack of antigenic differences among the strains. Control experiments showed no reactivity in any of the three serological techniques between preimmune sera and antigens nor between antisera and growth media.

An additional qualitative immunological method, growth inhibition of colonies on agar by antiserum discs, was utilized for strain comparison. The mechanism of growth inhibition by this technique has been shown by Stanbridge and Hayflick (22) to differ from MI in that heat-labile serum factors are not required. The growth of all strains was inhibited to an approximately equal degree by reference antiserum discs. Thus, by the four immunological methods employed, no differences could be discerned between any of the strains of *M. pneumoniae* tested, irrespective of virulence.

DISCUSSION

Comparison of homologous pairs of virulent and attenuated or avirulent strains of *M. pneumoniae*, derived by repeated passages of parent strains in artificial media or in a susceptible host, was intended to yield information about organism factors associated with virulence. One goal of the present study was the identification of an easily tested virulence marker to replace the more complicated technique of pathogenicity testing in hamsters. Although this need was not fully met, two markers (loss of both hemadsorption and aggregated growth in broth) were found to be associated with a total loss of virulence. These alterations were highly stable and may represent mutations.

Variations among *M. pneumoniae* strains have been noted by others who found differences in streptomycin sensitivity (9), histological (8) and clinical (7) pathogenicity, and growth characteristics related to temperature sensitivity, growth on glass, and anaerobiasis (23). A second goal of the present study was to determine the other variations that might exist between strains of *M. pneumoniae* and the techniques whereby variations might best be discerned. Strain variations were revealed utilizing seven markers: pathogenicity, morphology in broth, growth curves and GT, peroxide formation, cytadsorption, glycolysis, and protein composition. The spectrum of phenotypic markers described and cited suggests that *M. pneumoniae* strain characteristics may be less uniform than previously suspected. Additional techniques, such as further antibiotic sensitivity testing or screening for the ability to metabolize

TABLE 2. *CF titers and relatedness between virulent and attenuated strain pairs of M. pneumoniae*

Antigen	Antiserum titers[a] and degree of relatedness[b]				
	Mac-K	Mac-H106	M129-B6	M129-M8	M129-B169
Mac-K	*64* *100%*	32 >*100%*			
Mac-H106	128 >*100%*	*32* *100%*			
M129-B6			*128* *100%*	128 *100%*	64 *100%*
M129-M8			128 *100%*	*128* *100%*	64 *100%*
M129-B169			64 *100%*	64 *100%*	*32* *100%*

[a] Reciprocal of serum-dilution end point (homologous titers in italics).
[b] Complete relatedness = 100%.

TABLE 3. *Metabolic inhibition titers and relatedness between virulent and attenuated strain pairs of M. pneumoniae*

Antigen	Antiserum titers and degree of relatednes[a]				
	Mac-K	Mac-H106	M129-B6	M129-M8	M129-B169
Mac-K	*256* *100%*	256 *100%*			
Mac-H106	512 *100%*	*256* *100%*			
M129-B6			*512* *100%*	ND[b]	16 *100%*
M129-M8			ND	ND	ND
M129-B169			512 *100%*	ND	*16* *100%*

[a] See Table 2.
[b] Not done.

a variety of carbohydrates, might be applied in a search for still other differences among strains. Such markers, if they vary in naturally occurring strains, would be particularly useful in epidemiological studies of *M. pneumoniae*-induced disease. Even the biological characteristics now used to identify *M. pneumoniae* strains may be found to vary, which suggests the need for caution when interpreting the results of single diagnostic tests.

The applicability of methods used in the present study for analyzing strain differences appears to vary considerably. For example, polyacrylamide gel electrophoresis, with the *p*H and buffer employed here, proved incapable of differentiating between strains although Razin and Rottem reported species-related differences among some mycoplasmas (17). Likewise, the tests of antigenic specificity, principally CF, MI, and ID, demonstrated complete cross-reactivity in spite of their high degree of effectiveness at differentiating be-

tween various mycoplasma species. These procedures and others that determine organism composition, for example deoxyribonucleic acid homology, may not prove fruitful in defining strain variations. However, techniques measuring biological activity, such as peroxide formation or cytadsorption, may prove more useful in strain differentiation and in furthering the understanding of *M. pneumoniae* growth, metabolism, pathogenicity, and epidemiology.

It has been estimated that *M. pneumoniae* is the agent responsible for 8 to 39% of pneumonias occurring in young adults (3). Information about the mechanism of pathogenicity of this organism is therefore especially desirable. The present work suggests that a phase of respiratory epithelial
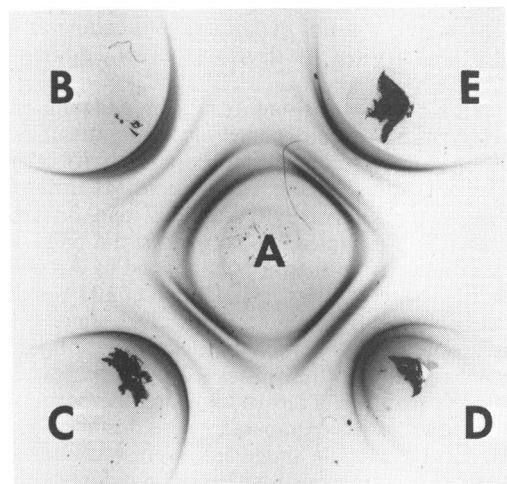

FIG. 5. *Double ID reaction between antiserum and antigens of virulent, attenuated, and avirulent strains of M. pneumoniae. (A) M129-B6 antiserum. (B) Mac-K, (C) Mac-H106, (D) M129-B169, and (E) M129-B6 antigens.*

cytadsorption is probably essential in a sequence of pathogenic events. However, strain Mac-K, which cytadsorbs, is not fully virulent, indicating that other steps, possibly involving a toxin, are also necessary. Complementary to the need for understanding the mechanism by which this organism produces disease is the need for development of effective prophylaxis. The inactivated vaccines which have been tested in human volunteers have not produced entirely satisfactory results (6). Although circulating antibodies could be induced in 50 to 75% of volunteers, the incidence of pneumonia after natural exposure or experimental challenge was reduced by only about 45% over that in control groups (16). Furthermore, illnesses more severe than in the control volunteers occurred in some vaccinated volunteers, suggesting vaccine-induced sensitization (19).

Recent work with the hamster model has shown that circulating antibodies induced by parenteral immunization with M. pneumoniae offer little protection against pneumonia from a challenge infection, in contrast to the more effective protection conferred by intranasal inoculation with a live attenuated strain (11). It appears, then, that effective immunity might best be produced locally, stimulated by a live vaccine strain which should cause no disease when administered intranasally. The only information about the human response to experimental infection with an attenuated strain suggests a reduced infectivity and antibody response (7). Of the three new attenuated strains now available, M129-B169 is easily grown in large quantities and is highly infectious yet avirulent in hamsters. This strain appears to be a promising vaccine candidate, and studies on its immunogenicity will be reported subsequently.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Archetti, I., and F. L. Horsfall. 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. 92:441–462.

2. Chanock, R. M., L. Hayflick, and M. F. Barile. 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Nat. Acad. Sci. U.S.A. 48:41–49.

3. Chanock, R. M. 1965. Mycoplasma infections of man. New Engl. J. Med. 273:1257–1264.

4. Clyde, W. A., Jr. 1963. Hemolysis in identifying Eaton's pleuropneumonia-like organism. Science 139:55.

5. Clyde, W. A., Jr. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. 92:958–965.

6. Clyde, W. A., Jr. 1969. Prevention of Mycoplasma pneumoniae disease. J. Infect. Dis. 120:255–257.

7. Couch, R. B., T. R. Cate, and R. M. Chanock. 1964. Infection with artificially propagated Eaton agent (Mycoplasma pneumoniae). Implications for development of attenuated vaccine for cold agglutinin-positive pneumonia. J. Amer. Med. Ass. 187:442–447.

8. Dajani, A. S., W. A. Clyde, Jr., and F. W. Denny. 1965. Experimental infection with Mycoplasma pneumoniae (Eaton's agent). J. Exp. Med. 121:1071–1086.

9. Eaton, M. D., and C. Liu. 1957. Studies on sensitivity to streptomycin of the atypical pneumonia agent. J. Bacteriol. 74:784–787.

10. Fernald, G. W., W. A. Clyde, Jr., and F. W. Denny. 1967. Factors influencing growth inhibition of Mycoplasma pneumoniae by immune sera. Proc. Soc. Exp. Biol. Med. 126:161–166.

11. Fernald, G. W. 1969. Immunologic aspects of experimental Mycoplasma pneumoniae infection. J. Infec. Dis. 119:255–266.

12. Kenny, G. W., and J. T. Grayston. 1965. Eaton pleuropneumonia-like organism (Mycoplasma pneumoniae) complement-fixing antigen: extraction with organic solvents. J. Immunol. 95:19–25.

13. Kim, K. S., W. A. Clyde, Jr., and F. W. Denny. 1966. Physical properties of human Mycoplasma species. J. Bacteriol. 92:214–219.

14. Lipman, R. P., and W. A. Clyde, Jr. 1969. The interrelationship of virulence, cytadsorption and peroxide formation in Mycoplasma pneumoniae. Proc. Soc. Exp. Biol. Med. 131:1163–1167.

15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

16. Mogabgab, W. J. 1968. Protective effects of inactive Mycoplasma pneumoniae vaccine in military personnel, 1964–1966. Amer. Rev. Resp. Dis. 97: 359–365.

17. Razin, S., and S. Rottem. 1967. Identification of Mycoplasma and other microorganisms by polyacrylamide-gel electrophoresis of cell proteins. J. Bacteriol. 94:1807–1810.

18. Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320–329.

19. Smith, C. B., W. T. Friedewald, and R. M. Chanock. 1967. Inactivated Mycoplasma pneumoniae vaccine. Evaluation in volunteers. J. Amer. Med. Ass. 199:353–358.

20. Sobeslavsky, O., B. Prescott, and R. M. Chanock. 1968. Adsorption of Mycoplasma pneumoniae to neuraminic acid receptors of various cells and possible role in virulence. J. Bacteriol. 96:695–705.

21. Somerson, N. L., B. E. Walls, and R. M. Chanock. 1965. Hemolysin of M. pneumoniae: tentative identification as a peroxide. Science 150:226–228.

22. Stanbridge, E., and L. Hayflick. 1967. Growth inhibition test for identification of Mycoplasma species utilizing dried antiserum-impregnated paper discs. J. Bacteriol. 93:1392–1396.

23. Steinberg, P., R. L. Horswood, and R. M. Chanock. 1969. temperature-sensitive (ts) mutants of Mycoplasma pneumoniae. I. In vitro biologic properties. J. Infec. Dis. 120: 217–224.

24. Takayama, K., D. H. MacLennan, A. Tzagoloff, and C. D. Stoner. 1966. Studies on the electron transfer system LXVII. Polyacrylamide gel electrophoresis of the mitochondrial transfer complexes. Arch. Biochem. Biophys. 114:223–230.