The Interrelationship of Virulence, Cytadsorption, and Peroxide Formation in *Mycoplasma pneumoniae** (34061)

RICHARD P. LIPMAN[1] AND WALLACE A. CLYDE, JR.[2]

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

Mycoplasma pneumoniae is an important cause of human lower respiratory tract infection and is the only mycoplasma associated definitely with disease in man. The mechanism by which this organism produces its pathology is unknown. Recently it was suggested that the quantity of peroxide secreted by *M. pneumoniae* and the tendency to adsorb to tracheal epithelium may be factors mediating virulence (1, 2). Since a reduction in *M. pneumoniae* virulence, with diminished pathogenicity for volunteers and hamsters, has been seen after subcultivation in artificial media (1, 2), an attempt was made to derive related pairs of virulent and attenuated strains by successive passage so that their biological characteristics might be compared. Using such pairs of virulent and attenuated strains, based on pathogenicity for hamsters, a quantitative measurement of peroxide formation and cytadsorption was therefore undertaken to assess the relatedness of these phenomena to virulence.

Material and Methods. Media. Cultivation utilized Hayflick's medium (5) containing 70% Difco PPLO agar or broth, 20% unheated horse serum, 10% aqueous yeast extract, 1% dextrose, penicillin G (1000 units/ml), and 0.004% phenol red indicator (broth only).

Mycoplasma strains. Organisms studied were derived from two parent strains of *M. pneumoniae*, both from patients with atypical pneumonia. M129-B6 had been isolated in this laboratory and passed six times in broth; Mac-K, isolated by M. D. Eaton and supplied by C. Liu, has been passed 78 times in chick embryo, 21 times on agar, and 123 times in broth.

A derivative of M129-B6 was produced by 169 serial passages in broth (M129-B169). Mac-K was passed eight successive times in hamsters (Mac-H106) by intranasal inoculation with 0.2 ml infected hamster lung as a 10% w/v suspension in 0.85% NaCl with 0.02 *M* phosphate buffer at pH 7.2 (PBS). It was then passed four times in broth. A single pool of each strain containing approximately 5×10^6 colony-forming units (CFU)/ml was prepared and used for all animal inoculations and *in vitro* studies. All strains were identified as *M. pneumoniae* by their glycolysis and by the antiserum disc inhibition technique (6). The pools were free of contamination by other microorganisms.

Measurement of virulence. Virulence was determined by the method of Dajani *et al.* (4). The severity of pneumonia produced in hamsters 14 days after intranasal inoculation of log-phase *M. pneumoniae* broth culture was studied histologically and the intensity of infection measured by counting CFU/gm of lung. Pneumonia was defined as a peribronchial or peribronchiolar round cell infiltration. A strain was considered virulent if an inoculation of approximately 5×10^5 CFU produced pneumonia in at least 80% of animals (7).

Cytadsorption and cytadsorption inhibition. The parent and derivative strains were

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[2] Research Career Development Awardee, USPHS Grant 2-KO3-AI-9676.

compared using the method of Sobeslavsky *et al.* (2) who demonstrated an equivalence between adsorption of erythrocytes and tracheal cells to *M. pneumoniae*. Colonies of *M. pneumoniae* on Hayflick's agar were overlaid with chicken red blood cells (RBC's), incubated 15 min at 37°, washed, and observed microscopically for degree of hemadsorption. Susceptibility to the effects of hemadsorption inhibitors was compared by treatment of the *M. pneumoniae* colony adsorption receptors with sialic acid (*N*-acetylneuraminic acid, General Biochemicals) or gastric mucin (Wilson Laboratories) in several concentrations prior to overlay with chicken RBC's. The inhibitory effect of receptor-destroying enzyme (RDE, Microbiological Associates, titer 1:800) on hemadsorption by the pairs of organisms was evaluated by pretreatment of the chicken RBC's with RDE (2).

Measurement of peroxide formation and inhibition. A modification of the technique of Sobeslavsky and Chanock was used (1). *M. pneumoniae* colonies grown for 5 days on 3-ml aliquots of Hayflick's agar in 60 × 15 mm plastic petri dishes were overlaid with 1 ml of a mixture of 3% thrice-washed sheep RBC's and 0.8% Difco Bacto-agar in PBS at 45°. Plates were then incubated at 37° in an atmosphere of 5% CO_2–95% air, and observed for hemolysis every 24 hr. To compare hemolysis produced by different strains in the presence of a peroxide inhibitor, dilutions of beef liver catalase (Worthington Biochemical Corp.) were incorporated into the blood-agar overlay. The hemolytic zone and colony diameters of 20 colonies of each strain were determined for each catalase dilution 48 hr after overlay using a microscope measuring engine reading directly in microns. For the purpose of strain comparison, peroxide formation was quantitatively expressed as the "hemolytic index": the ratio of hemolytic zone diameter to colony diameter.

Results. Virulence of parent and derived strains. The effects of intranasal inoculation of hamsters with the four strains are summarized in Table I. All animals became infected. The intensity of infection, measured by the mean CFU/gm of lung, was the same for all strains, within the range of experimental error.

Histologic pneumonia was produced by Mac-K in 4/9 (44%) of animals; the mean severity of pneumonia as defined in Table I was 1.0, or mild. Its derivative, Mac-H106, produced pneumonia in 12/12 animals, with a mean severity of 2.3 or moderate-to-extensive. Thus, a fully virulent strain was derived from an attenutated strain by repeated passage in a susceptible host. M129-B6 produced pneumonia in 6/6 animals, with a mean severity of 2.3. Its derivative, M129-B169, was found to be completely attenuated, causing no pneumonia in 9 animals. Having two sets of *M. pneumoniae* pairs, each consisting of a virulent and attenutated strain of common

TABLE I. Effects of *M. pneumoniae* Strains on Hamsters.

	Strain[a]			
	Mac		M129	
Inoculation result	K	H106	B6	B169
Infection				
Positive/no. inoculated	9/9	12/12	6/6	9/9
Mean CFU/g lung	6.0×10^5	6.5×10^5	2.4×10^5	3.2×10^5
Pneumonia				
Positive/no. inoculated	4/9	12/12	6/6	0/9
Severity[b]	1.0[c]	2.3	2.3	—

[a] For derivation, see text.

[b] 1 = One area of definite pneumonia; 2 = At least two areas of definite pneumonia; 3 = All bronchi involved.

[c] Mean severity in pneumonia-positive animals.

TABLE II. Comparative Hemadsorptive Activity of Virulent and Attenuated Strains: Effect of Interfering Agents.

	Hemadsorption[a]			
	Mac		M129	
Inhibitor	K (Attenuated)[b]	H106 (Virulent)	B6 (Virulent)	B169 (Attenuated)
None	++++	++++	++++	0
RDE				
1:16	+++	+++	+++	0
1:8	+	+	+	0
1:4	0	0	0	0
Sialic acid				
1%	+++	++++	++++	ND[c]
2%	+	0	+++	ND
4%	0	0	0	ND
Gastric mucin				
1%	+	+	+	ND
3%	+	+	+	ND
5%	0	0	0	ND

[a] + = 2–5 RBC's adherent to every colony; ++ = less than 2/3 colony obscured by RBC's; +++ = more than 2/3 colony obscured; ++++ = entire colony obscured.
[b] See Table I.
[c] Not done.

origin, presented the opportunity to evaluate the quantitative relationship between virulence, hemadsorptive activity, and hemolytic activity.

Hemadsorptive activity of virulent and attenuated strains. When homologous strain pairs were compared by measuring their hemadsorptive activity, one attenuated and both virulent strains exhibited the same marked degree of hemadsorption, as summarized in Table II. The other attenuated strain, M129-B169, showed no hemadsorption on repeated testing with chicken, human, sheep, guinea pig, horse, or monkey RBC's. Hemadsorption of 1–10 hamster RBC's to colonies of this strain was occasionally seen. The avidity of the three hemadsorbing strains for RBC's was then compared by measuring their residual degree of binding after either exposure of colonies to sialic acid or gastric mucin, or pretreatment of RBC's with RDE. As seen in Table II, all strains demonstrated the same degree of hemadsorption in the presence of inhibitors except for M129-B6, which was relatively resistant to sialic acid. These observations confirm the findings of Sobeslavsky *et al.* (2) that hemadsorption by *M. pneumoniae* colonies can be blocked by treatment of their receptor sites with substances containing *N*-acetylneuraminic acid or by pretreatment of RBC's with reagents which destroy neuraminic acid-containing binding sites. The present work, however, indicates that possession of intense hemadsorption is not a quality unique to virulent strains of *M. pneumoniae* and that homologous virulent and attenuated pairs may hemadsorb to an equal degree. Nevertheless, the loss of hemadsorbing activity appears to be inconsistent with virulence, and may even preclude it. Also, as suggested by the data for hemadsorption inhibition by sialic acid, it is possible that equally virulent strains may vary in cytadsorptive activity.

Hemolytic acitvity of virulent and attenuated strains. The hemolysis of RBC's overlaid on colonies of *M. pneumoniae* has been shown to be due to peroxide elaborated by that organism (8). To assess the relative ability of the virulent and attenuated strains

TABLE III. Comparative Hemolytic Activity of Virulent and Attenuated Strains: Effect of Catalase.

	Hemolytic index[a]			
	Mac		M129	
Catalase concentration[b]	K (Attenuated)	H106 (Virulent)	B6 (Virulent)	B169 (Attenuated)
0	10.23 ± 1.18	9.01 ± 0.89	6.01 ± 1.48	6.58 ± 0.78
2000 U	ND[c]	ND	4.39 ± 0.86	4.22 ± 0.62
4000 U	3.63 ± 0.75	3.00 ± 0.42	ND	ND

[a] Mean ratio of hemolytic zone to colony diameter ± 1 standard deviation.
[b] Units of catalase per milliliter of overlay.
[c] Not done.

to produce peroxide, the magnitude of their hemolytic activity, as well as the quantity of enzymes necessary to block this activity, were measured. As summarized in Table III there were no significant differences in the hemolytic activities of corresponding virulent and attenuated pairs. However, both strains of Mac had a higher hemolytic index than did the M129 pair, indicating that quantitative differences in peroxide formation may exist between strains of *M. pneumoniae,* but are unrelated to virulence or degree of attenuation.

Further evidence that virulence does not depend solely on peroxide formation is suggested by the equivalent amounts of catalase required to effect equal inhibition of hemolysis by homologous pairs (see Table III). Other experiments conducted to determine the minimum concentration of catalase necessary to block all visible hemolysis likewise showed that hemolysis by homologous pairs underwent complete inhibition in the presence of identical concentrations of catalase. Control preparations utilizing catalase inactivated by heating at 56° for 20 min showed no such inhibition.[3]

Discussion. The phenomenon of virulence variation must be considered when *M. pneumoniae* strains are undergoing evaluation for pathogenicity-associated markers. The existence of this variable could be inferred from studies in which laboratory attenuation appeared to occur (3, 4); it is possible that variations in virulence also occur among wild strains. Such a variable might be of great importance in antigen analysis, in the stimulation of antibodies in experimental animals, in human vaccine production, and in the use of strains to challenge vaccine protection. Virulence is an *in vivo* phenomenon; previous discussions relating to it the characteristics of peroxide formation (9) and cytadsorption (10), demonstrated *in vitro,* have not explored the possibility of variations in viru-

[3] The use of peroxidase to substantiate this quantitative study of peroxide formation appeared less specific on theoretical grounds, since peroxidase catalyzes the oxidation of substrates by peroxide to a far greater extent than it acts upon peroxide directly. Introduction of the variable of substrate oxidation, not a factor when catalase is used, would make the measurement of hemolytic activity in the presence of peroxidase a less accurate indicator of peroxide formation. Experiments using an overlay containing horseradish peroxidase (Nutritional Biochemicals) rather than catalase produced extremely variable hemolytic zones from colony to colony. Whereas a plaque of intact RBC's was ordinarily seen over the colonies of control and catalase preparations, possibly indicating an endogenous protective mechanism against the effects of peroxide, this was absent in the presence of peroxidase. Similarly absent was the characteristic greenish band of RBC's at the outer margin of hemolysis. Instead, a thin ring of intact RBC's persisted around every colony in the midst of the clear hemolytic zone, in the approximate position otherwise occupied by the missing plaque margin. These striking departures from the appearance of both control and catalase preparations suggest the necessity for caution in interpreting measurements of hemolytic activity of *M. pneumoniae* based on peroxidase inhibition.

lence of the *M. pneumoniae* strains in question. Using the hamster model, marked differences in virulence have now been demonstrated in two well-characterized strains. It has then proved possible to derive a virulent strain from the attenuated parent by repeated passage in a susceptible host, and to attenuate a virulent strain by repeated passage in artificial media. Comparison of peroxide formation and cytadsorption by these pairs has shown no greater activity in the virulent strains, although the isolated loss of cytadsorption was associated with a loss of virulence in one case. Thus, cytadsorptive activity and peroxide formation can vary independently. The theory that pathogenesis follows a sequence of organism adsorption to respiratory epithelium and then direct damage by peroxide (2) is plausible, but the present studies indicate that these, either singly or together, are not entirely responsible for virulence.

It is suggested, therefore, that while this sequence of cytadsorption and peroxide formation may be necessary for *M. pneumoniae* strain virulence in a susceptible host, other important direct mediators of virulence exist and must also be present simultaneously. Accordingly, certain other biological characteristics of these virulent and attenuated pairs have been the object of an investigation to be reported subsequently.

An apparent difference between virulent strains in peroxide formation and cytadsorption has been noted. These, along with virulence, now provide three markers for strain variation among *M. pneumoniae* isolates, and raise the possibility that other characteristics may also vary. Although of doubtful value for taxonomy, such subtyping may be useful in the further understanding of *M. pneumoniae* biology, host–parasite interaction, and epidemiology.

Summary. A study of the relationship between virulence, peroxide formation, and cytadsorption in *M. pneumoniae* was undertaken. Two pairs of homologous virulent and attenuated strains were derived by passage of parent strains in animals and artificial media. Comparison of their cytadsorptive activity and peroxide formation by measurement of hemadsorption and hemolysis revealed variations between heterologous strains, but no differences consistently related to virulence. The existence of additional mediators of virulence is suggested.

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