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Adsorption of *Mycoplasma pneumoniae* to Neuraminic Acid Receptors of Various Cells and Possible Role in Virulence

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Monkey, rat, and chicken tracheal epithelial cells, as well as monkey, rat, guinea pig, and chicken erythrocytes, adsorbed firmly to colonies of *Mycoplasma pneumoniae* and *M. gallisepticum*. Colonies of *M. pulmonis* also adsorbed erythrocytes but with less avidity than *M. pneumoniae* or *M. gallisepticum*; unlike the latter organisms, *M. pulmonis* did not adsorb tracheal epithelial cells. Colonies of *M. orale* type 1 and *M. orale* type 3 adsorbed only chicken red cells. Other mycoplasma species tested, including four of human origin and one of animal origin, did not adsorb red cells or epithelial cells. *M. pneumoniae* and *M. gallisepticum* appeared to attach to erythrocytes or tracheal epithelial cells by neuraminic acid receptors on these cells, whereas *M. orale* types 1 and 3 and *M. pulmonis* seemed to utilize another type or other types of receptors. Pretreatment of red cells or tracheal epithelial cells with receptor-destroying enzyme, neuraminidase, or influenza B virus removed the adsorption receptors for *M. pneumoniae*. Similarly, pretreatment of *M. pneumoniae* colonies with neuraminic acid-containing materials prevented adsorption of erythrocytes or respiratory tract cells. The adsorption sites on *M. pneumoniae* were specifically blocked by homologous but not heterologous antisera. This property made it possible to study the nature of the mycoplasma adsorption sites by testing the capacity of different fractions of the organism to block the action of adsorption-inhibiting antibodies. Such studies suggested that the mycoplasma binding sites were probably lipid or lipoprotein in nature. The glycerophospholipid hapten was implicated as one such site, since this serologically active hapten blocked the action of hemadsorption-inhibiting antibodies in *M. pneumoniae* rabbit antiserum. The affinity of *M. pneumoniae* for respiratory tract epithelium, unique among the mycoplasmas that infect man, may play a role in virulence, since this type of attachment provides an unusual opportunity for peroxide, secreted by the organism, to attack the tissue cell membrane without being rapidly destroyed by catalase or peroxidase present in extracellular body fluids.

Mycoplasma pneumoniae is the most virulent of the mycoplasmas that infect man (3). It has been suggested that its virulence is due, in part, to a peroxide hemolysin secreted by this organism (7, 16). Non-*M. pneumoniae* mycoplasmas of man, except for the "T" strains, also generated such a peroxide hemolysin; however, the extent of peroxide secretion by these organisms was less than that of *M. pneumoniae*. Since nonpathogenic mycoplasmas secrete peroxide, it would appear that one determinant of virulence of *M. pneumoniae* is more likely the amount of peroxide secreted rather than peroxide generation per se (Sobeslavsky and Chanock, *in press*).

Recently hemadsorption of red blood cells by *M. pneumoniae* was described (R. A. Del Giudice and R. Pavia, Bacteriol. Proc., p. 71, 1964). When guinea pig erythrocytes suspended in saline were applied to colonies of *M. pneumoniae* growing on agar, red blood cells firmly adhered to the colonies. This phenomenon suggested to us that such direct contact between mycoplasma and red cells could facilitate diffusion of peroxide to the cell membrane and thus enhance injury of affected cells.

In view of these findings, it was of interest to

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know whether *M. pneumoniae* could also adsorb to cells of other tissues, namely, those of the respiratory tract, and if so, whether other mycoplasmas of man possess the same capacity. Such information would have relevance to the pathogenesis of respiratory disease caused by *M. pneumoniae*. In addition, we were interested in defining the nature of the adsorption mechanism of those mycoplasmas which attach to host cells.

MATERIALS AND METHODS

Organisms. Seven species of mycoplasmas which infect man and three species of mycoplasmas which infect animals were studied. All strains employed were subjected to multiple passages in mycoplasma media and were cloned three times prior to use in this study. Mycoplasmas derived from man were *M. pneumoniae* (strains FH, PI-1428), *M. hominis* (strains PG-21, DC-63, V-2785), *M. orale* type 1 (strains CH-19299, LJ-75164, LJ-72738), *M. orale* type 2 (strains CH-20247, DC-1600), *M. orale* type 3 (strain DC-1114), *M. salivarium* (strains PG-20, DC-585, Buccal), and *M. fermentans* (strain PG-18). Mycoplasmas derived from animals were *M. gallisepticum* (strain PG-31), *M. pulmonis* (strains PG-22, 880, Negroni), and *M. neurolyticum* (strain PG-28).

Media. Medium used for the growth of *M. hominis*, *M. orale* type 1, *M. orale* type 2, *M. orale* type 3, *M. salivarium*, and *M. neurolyticum* was the agar medium described by Chanock, Hayflick, and Barile, except that agamma-globulin horse serum was substituted for normal horse serum (4). This medium, supplemented with 1% glucose, was used to grow *M. pneumoniae*, *M. fermentans*, *M. gallisepticum*, and *M. pulmonis*. *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. gallisepticum*, *M. pulmonis*, and *M. neurolyticum* were propagated under aerobic conditions, whereas *M. orale* type 1, *M. orale* type 2, *M. orale* type 3 (*in preparation*), and *M. salivarium* were grown in an atmosphere of 5% CO_2-95% N_2. Colonies were well formed after 3 to 5 days of incubation at 37 C, at which time they were overlaid with 0.5 ml of a 0.5% suspension of red blood cells (monkey, guinea pig, rat, or chicken) that had been washed three times in saline. When tracheal or renal cell adsorption was tested, mycoplasma colonies were overlaid with 0.5 ml of a suspension of tissue cells (approximately 100,000 cells/ml) that had been washed three times in saline. Respiratory epithelial cells were prepared by scraping the tracheal mucous membranes derived from vervet monkeys, rats, or chickens. These cells were dispersed by vigorous to and fro pipetting. Suspensions of monkey renal cells were prepared by trypsinization of kidney tissues. Mycoplasma colonies (overlaid with red blood cells or tissue cells) were incubated for 30 min at room temperature, and unadsorbed cells were removed by gentle washing with saline; vigorous washing often led to detachment of colonies from the agar surface. Adsorption was examined by low-power microscopy (100 ×).

Inhibition of hemadsorption or cell adsorption. The nature of the cell adsorption receptors was examined by exposing red cells or tracheal epithelial cells to the following reagents or conditions: heat, 56 C for 30 min; trypsin, 0.25% at 37 C for 30 min; KIO_4, 0.003 to 0.011 M at room temperature for 30 min; receptor-destroying enzyme (RDE) (Microbiological Associates, Inc., Bethesda, Md.), 1 unit at 37 C for 30 min; purified neuraminidase from *Vibrio cholerae* (General Biochemicals, Chagrin Falls, Ohio), 1 to 10 units at 37 C for 30 min; influenza B virus (Taiwan, 1962), 1 to 4 hemagglutinin (HA) units at 37 C for 30 min; parainfluenza virus type 3, 16 HA units at 37 C for 30 min, and *M. pneumoniae* (strain FH), 2% suspension at 37 C for 30 min. One volume of 0.5% red blood cells or tracheal epithelial cells (100,000 cells/ml) was mixed with one volume of the reagent to be tested, and the mixture was incubated, with occasional shaking, at 37 C. After 30 min of incubation, the cells were washed and resuspended to original volume in saline. Treated cells (0.5 ml) were then pipetted onto the agar surface which contained mycoplasma colonies. RDE and neuraminidase were diluted in calcium acetate saline (CAS), *p*H 6.2 (1), whereas the other reagents were diluted in phosphate-buffered saline (PBS), *p*H 7.2.

The nature of adsorption receptors on *M. pneumoniae* was examined by exposing colonies to the following reagents (0.5 ml volume) or conditions: heat, 56 C for 1 to 4 min; ultraviolet light (General Electric G-30T8, 30 w), 44 $\mu w/cm^2$ at room temperature for 2 to 2.5 min; CAS, *p*H 4.5 to 5.5 at room temperature for 30 min; formaldehyde, 0.1 to 0.4% at room temperature for 60 min; merthiolate, 0.01 to 0.1% at room temperature for 60 min; trypsin, 0.25% at 37 C for 30 min; KIO_4, 0.011 M at room temperature for 1 to 3 hr; RDE, 8 units at 37 C for 30 min; gastric mucin (Nutritional Biochemicals Corp., Cleveland, Ohio), *p*H 6.2, 0.5 to 1% at 37 C for 3 hr; *N*-acetylneuraminic acid (General Biochemicals), *p*H 6.2, 1 to 4% at 37 C for 3 hr; actinomycin D (Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.), 0.1 to 1 μg/ml at 37 C for 24 hr; puromycin dihydrochloride (Nutritional Biochemicals Corp.), 1 to 200 μg/ml at 37 C for 24 hr; and aminonucleoside (puromycin, Nutritional Biochemicals Corp.), 1 to 4,000 μg/ml at 37 C for 24 hr. After the indicated treatment, colonies were washed once with saline, and 0.5 ml of a 0.5% suspension of guinea pig erythrocytes or 0.5 ml of tracheal epithelial cells (100,000 cells/ml) was applied. For dilution of RDE, gastric mucin, neuraminelactose, and *N*-acetylneuraminic acid, CAS was used while other reagents were diluted in PBS. The *p*H of neuraminelactose and *N*-acetylneuraminic acid was adjusted with 1 N NaOH to 6.2 prior to dilution.

Hemadsorption inhibition by specific antisera. To demonstrate the specificity of the adsorption reaction, *M. pneumoniae* colonies were preincubated with dilutions of potent specific antisera to the following mycoplasmas: *M. pneumoniae*, *M. hominis*, *M. orale* type 1, *M. orale* type 2, *M. salivarium*, *M. fermentans*, *M. gallisepticum*, and *M. pulmonis*. To avoid nonspecific reactions with constituents of growth media, rabbit antisera were prepared from strains adapted to growth in rabbit infusion broth supplemented with

rabbit serum and yeast dialysate, whereas mule antiserum for *M. pneumoniae* was prepared from a strain adapted to growth in mule infusion broth supplemented with mule serum and yeast dialysate (19; T. R. Carski, *personal communication*).

Twofold dilutions (0.5 ml) of immune sera in PBS were applied to *M. pneumoniae* colonies on agar, and the cultures were incubated for 1 hr at 37 C. The cultures were then washed once with saline, and 0.5 ml of a 0.5% suspension of guinea pig red blood cells or 0.5 ml of tracheal epithelial cells (100,000 cells/ml) was applied to the agar surface.

Gel filtration of immune sera. To identify the immunoglobulins responsible for hemadsorption inhibition, hyperimmune rabbit, guinea pig, and mule *M. pneumoniae* antisera were subjected to gel filtration using Sephadex G-200 gel (Pharmacia, Inc., New Market, N.J.). A 3-ml sample of serum was applied to a column 700 mm in length and 20 mm in width, and eluates were collected in 3-ml volumes as described elsewhere (2). Protein concentrations of eluates from Sephadex chromatogram were determined by the Lowry method (10). The eluates containing 19*S* or 7*S* globulins were pooled, dialyzed, lyophilized, and reconstituted to the original volume of the serum. Fractionation of the sera was kindly performed by Joseph Bellanti.

Chemical extraction and chromatographic separation of fractions of M. pneumoniae. Purified suspensions of *M. pneumoniae* were prepared from organisms grown on a glass surface. Prior to their removal from the glass surface, the organisms were washed extensively to remove constituents of the growth medium (15). To determine which components of *M. pneumoniae* were involved in the adsorption reaction, fractions of the organism were prepared by chemical and chromatographic separation described previously (12). Following chemical extraction of *M. pneumoniae* suspensions, one lipid fraction, two protein fractions, and three polysaccharide fractions were obtained, while Sephadex G-25 chromatography of the soluble supernatant fluid of disrupted organisms (10 kc for 30 min) yielded four fractions. The Sephadex fractions contained mostly protein and some polysaccharides. Sephadex G-25 fraction 1 also contained approximately 15% lipid by weight. Lipid was extracted from this fraction by treatment with a 95% ethyl alcohol-ammonium hydroxide (100:2) mixture.

The total lipid that was chemically extracted from *M. pneumoniae* and which comprised approximately 20% of the organism by weight was further fractionated on a silicic acid column by a method described previously (13). The fractions eluted from the silicic acid column with benzene-hexane (6:94, 1:4, or 6:4), benzene, $CHCl_3$-benzene (3:1), $CHCl_3$, $CHCl_3$-CH_3OH (20:1, 9:1, 4:1, 1:1, or 1:20), or ether were dried in vacuo, taken up in a small volume of propylene glycol, and suspended in isotonic saline to a concentration of 0.1% by weight. The fraction which was eluted from the column with a 9:1 mixture of $CHCl_3$-CH_3OH possessed both high-complement-fixing activity and metabolism-inhibiting antibody blocking activity, whereas the other fractions were without either activity (11). This active material appeared to be a phospholipid which contained saturated and unsaturated fatty acids linked to glycerol by ester bonds. In addition, it was free of detectable sugars and nitrogen (Prescott, Chernick, and Chanock, *unpublished data*). When inoculated into animals, the glycerophospholipid failed to stimulate antibody and thus appeared to be a hapten.

Test of inhibition of hemadsorption inhibition. This test was devised to measure the capacity of *M. pneumoniae* or its fractions to block hemadsorption-inhibiting antibodies by combining with such antibodies. Various concentrations of *M. pneumoniae* or fractions of this organism (0.25-ml volume) were mixed with an equal volume of *M. pneumoniae* antiserum diluted to contain 8, 4, 2, or 1 unit of hemadsorption-inhibiting antibody. Serum dilutions mixed with a suspension of *M. gallisepticum* or *M. fermentans* served as controls. The mixtures were incubated with occasional shaking for 1 hr at 37 C and applied to *M. pneumoniae* colonies growing on agar medium. Subsequently, these agar plates were incubated at 37 C for 1 hr, then washed with saline, and 0.5 ml of a 0.5% suspension of guinea pig red cells or 0.5 ml of tracheal epithelial cells (100,000/ml) in saline was applied to the colonies. A blocking effect was considered significant when the test material blocked the inhibitory effect of 4 to 8 units of hemadsorption-inhibiting antibody.

Complement fixation and inhibition of metabolism-inhibiting antibody tests. The *M. pneumoniae* suspensions and fractions which were tested for hemadsorption inhibition blocking activity were also tested for both complement-fixing activity and metabolism-inhibiting antibody blocking activity by methods described previously (14).

Immunization of animals with M. pneumoniae fractions. Antisera to the fractions of *M. pneumoniae* were prepared in rabbits and guinea pigs according to immunization methods described previously (14).

RESULTS

Adsorption of erythrocytes or epithelial cells to mycoplasma colonies. Results of adsorption experiments are summarized in Table 1. Guinea pig, monkey, rat, or chicken erythrocytes adsorbed to colonies of *M. pneumoniae* or *M. gallisepticum* (Fig. 1). Epithelial cells from monkey, rat, or chicken trachea, as well as monkey kidney cells, were adsorbed in a similar fashion (Fig. 2). *M. pulmonis* colonies also adsorbed each of the red blood cell types tested, but adsorption was less extensive than with *M. pneumoniae* or *M. gallisepticum;* however, *M. pulmonis* failed to adsorb trachea or kidney cells. Colonies of *M. orale* type 1 and *M. orale* type 3 adsorbed only chicken erythrocytes. The other mycoplasmas tested did not adsorb red blood cells or epithelial cells.

The extent of adsorption to *M. pneumoniae* was approximately the same whether the tests were performed at 37 C, room temperature, or 4 C.

TABLE 1. *Adsorption of erythrocytes or epithelial cells to mycoplasmas*

Mycoplasma	Adsorption to indicated type of cell					
	Erythrocyte				Epithelial cell	
	Monkey, rat, or guinea pig		Chicken		Monkey trachea, monkey kidney, rat trachea, or chicken trachea	
	Untreated	RDE-treated[a]	Untreated	RDE-treated[a]	Untreated	RDE-treated[a]
M. pneumoniae	++[b]	0 or +[c]	++	0	++	0 or +[c]
M. gallisepticum	++	0	++	0	++	0 or +[c]
M. pulmonis	+	+	+	+	0	0
M. orale 1	0	0	+	++	0	0
M. orale 3	0	0	+	++	0	0
M. orale 2, *M. salivarium*, *M. hominis*, *M. fermentans* or *M. neurolyticum*	0	0	0	0	0	0

[a] Eight units of RDE used.
[b] Maximal adsorption.
[c] RDE treatment (32 units) did not completely remove receptors from rat cells.

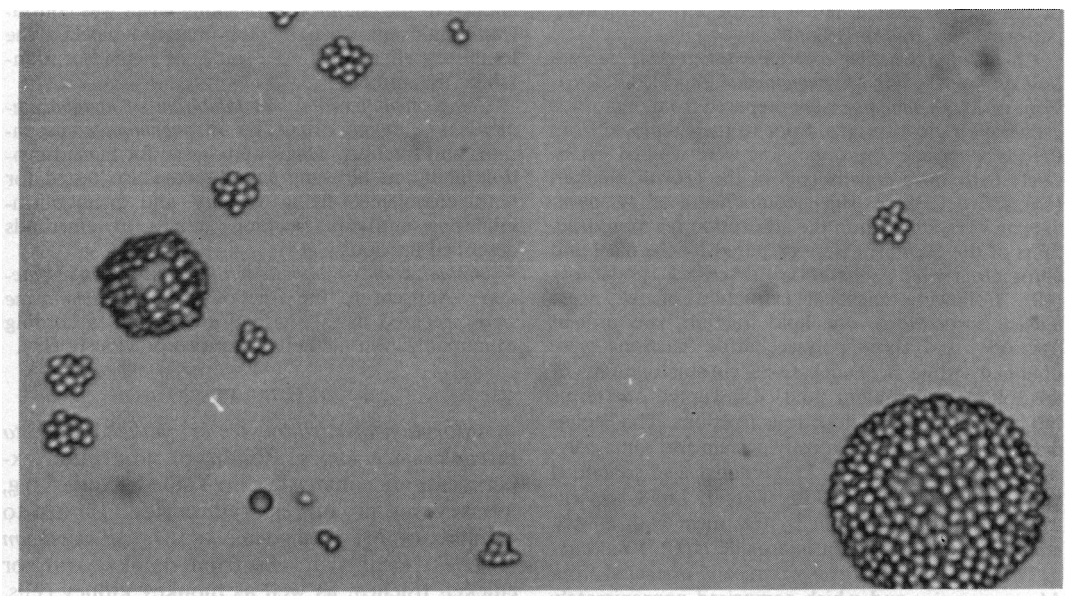

FIG. 1. *Adsorption of guinea pig erythrocytes to colonies of Mycoplasma pneumoniae.*

Adsorption began shortly after erythrocytes or epithelial cells were added to the mycoplasma colonies; however, maximal adsorption required 15 to 30 min. After 24 hr of incubation at 37 C, there was no evidence that the adsorbed cells were released from the mycoplasma colonies. All aspects of the tracheal cell surface participated in the adsorption reaction. Adsorption via the nonciliated surface occurred as often as that involving the ciliated border.

Hemadsorption was influenced by age and density of mycoplasma colonies. Young colonies, i.e., 3 to 7 days old, adsorbed more effectively than older colonies. Inhibition of hemadsorption was observed when colony density was higher than 10^4 colony-forming units (CFU) per 35-mm diameter agar plate culture. For this reason, although the mechanism of the "crowding effect" was not understood, tests for hemadsorption or cell adsorption were performed with cultures containing approximately 100 to 200 CFU.

Mechanism of hemadsorption or cell adsorption

by M. pneumoniae. Eyrthrocytes or epithelial cells treated with RDE lost their capacity to adsorb to *M. pneumoniae* or *M. gallisepticum* colonies (Table 1). However, such treatment did not affect adsorption of erythrocytes to *M. pulmonis* colonies. In the case of *M. orale* type 1 and *M. orale* type 3, RDE treatment of chicken red blood cells further enhanced their adsorptive efficiency.

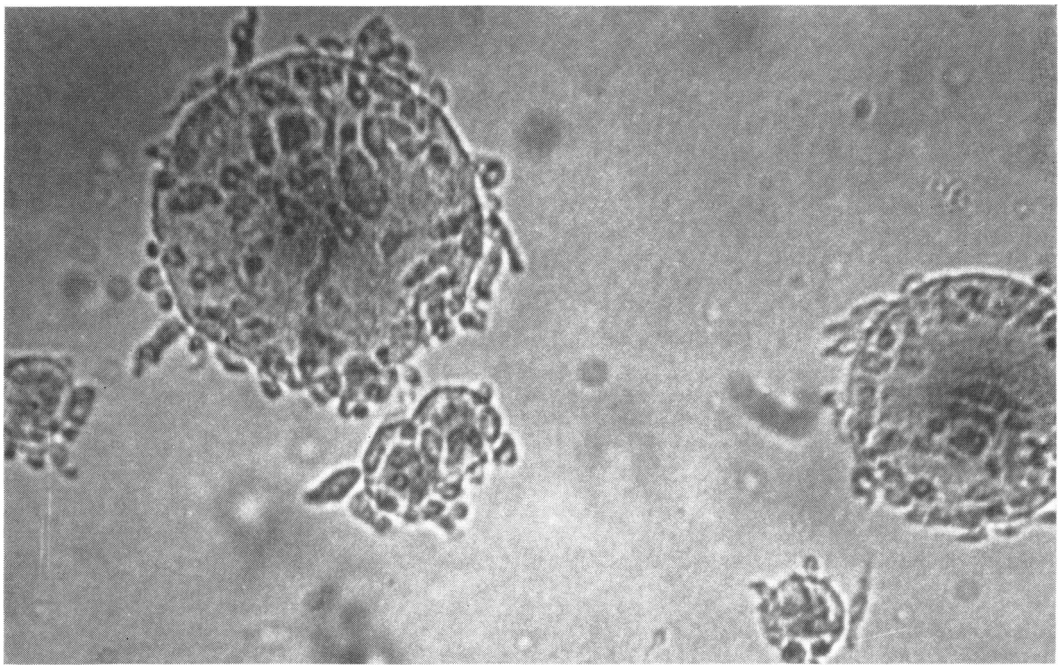

FIG. 2. *Adsorption of vervet monkey tracheal epithelial cells to colonies of Mycoplasma pneumoniae.*

TABLE 2. *Effect of different treatments on guinea pig erythrocyte or monkey tracheal epitheal cell receptors for M. pneumoniae*

Treatment of guinea pig erythrocytes or monkey tracheal cells			Adsorption following indicated treatment	
Reagent or condition	Incubation	Concn	Guinea pig erythrocytes	Monkey tracheal cells
Influenza B virus (Taiwan, 1962)	37 C for 30 min	4 HA[a] units	0	0
		2 HA units	+	0
		1 HA unit	++[b]	++
Receptor-destroying enzyme (*Vibrio cholerae* filtrate)	37 C for 30 min	1 unit[c]	0	0
Neuraminidase (purified from *V. cholerae*)	37 C for 30 min	10 units	0	0
		5 units	+	0
		1 unit	++	++
KIO_4	Room temperature for 30 min	0.011 M	0	0
		0.006 M	+	+
		0.003 M	++	++
Parainfluenza virus type 3	37 C for 30 min	16 units	++	++
Trypsin	37 C for 30 min	0.25%	++	++
Heat	56 C for 30 min		++	++
M. pneumoniae	37 C for 30 min	2%	++	++

[a] HA, hemagglutinin.
[b] Maximal adsorption.
[c] Highest dilution of RDE which rendered guinea pig erythrocytes nonagglutinable by 8 HA units of influenza B virus (Taiwan).

These results suggest that *M. pneumoniae* and *M. gallisepticum* attach to erythrocytes or epithelial cells by neuraminic acid receptors, whereas *M. orale* type 1, *M. orale* type 3, and *M. pulmonis* utilize another type of receptor or other types of receptors.

The nature of the cell receptor for *M. pneumoniae* was studied in greater detail by treating guinea pig erythrocytes or monkey tracheal epithelial cells with KIO_4, purified neuraminidase from *V. cholerae*, or with influenza B virus. Each of these treatments removed cell receptors for *M. pneumoniae* (Table 2). In addition, pretreatment of *M. pneumoniae* colonies with neuraminic acid, containing substances such as gastric mucin or neuraminelactose or *N*-acetylneuraminic acid, blocked their adsorptive activity (Table 3). These results further support the contention that neuraminic acid on the cell surface was responsible for the adsorption reaction with *M. pneumoniae*. Pretreatment of red cells or epithelial cells with heat, trypsin, parainfluenza 3 virus, or *M. pneumoniae* failed to remove receptors (Table 2).

Formaldehyde, merthiolate, and actinomycin D affected the adsorption sites on *M. pneumoniae*, since colonies treated with appropriate concentrations of these compounds failed to adsorb red cells or epithelial cells (Table 3). Concentrations of these compounds that inhibited adsorption also caused death of the surface layer of organisms, since colonies did not develop on agar rubbed with a push block derived from the treated culture. Appropriate concentrations of KIO_4, trypsin or puromycin, or treatment with heat or *p*H 4.5 (calcium acetate saline) inhibited the adsorptive capacity of *M. pneumoniae* colonies.

TABLE 3. *Effect of different treatments on adsorption receptors of M. pneumoniae*

Treatment of *M. pneumoniae* colonies			Adsorption of guinea pig erythrocytes or monkey tracheal cells
Reagent or condition	Incubation	Concn	
Formaldehyde	RT[a] for 60 min	0.4%	0
		0.2%	+
		0.1%	++[b]
Merthiolate	RT for 60 min	0.1%	0
		0.01%	+
KIO_4	RT for 120 min	0.011 M	0
	RT for 60 min		+
Trypsin	37 C for 30 min	0.25%	0
RDE	37 C for 30 min	8 units[c]	++
Heat	56 C for 3 min		0
	56 C for 2 min		+
	56 C for 1 min		++
Ultraviolet light	RT for 2.5 min	44 μw/cm^2	0
	RT for 2 min		++
Calcium acetate saline	RT for 30 min	*p*H, 4.5	0
		*p*H, 5.0	+
		*p*H, 5.5	++
Actinomycin D[d]	37 C for 24 hr	0.5–1 μg/ml	0
		0.1–0.4 μg/ml	++
Puromycin dihydrochloride[d]	37 C for 24 hr	3–5 μg/ml	0
		1 μg/ml	++
Aminonucleocide (puromycin)[d]	37 C for 24 hr	1–4,000 μg/ml	++
N-acetyl neuraminic acid, *p*H 6.2	37 C for 3 hr	4%	0
		2%	+
		1%	++
Neuramine lactose, *p*H 6.2	37 C for 3 hr	1%	0
		0.5%	+
Gastric mucin[e]	37 C for 3 hr	4%	0
		3%	+
		2%	++

[a] RT, room temperature.
[b] Maximal adsorption.
[c] One unit of RDE removed receptors for *M. pneumoniae* adsorption from guinea pig erythrocytes.
[d] Not tested with monkey tracheal cells.
[e] Same results obtained when heated 56 C for 30 min.

In these instances, viable organisms were still present in the colonies after treatment as indicated by the "agar push block" technique; however, no attempt was made to determine the quantitative aspects of mycoplasma inactivation. The adsorptive activity of *M. pneumoniae* was not affected by 8 units of RDE nor by 4,000 μg of aminonucleoside per ml.

Inhibition of adsorption activity of M. pneumoniae by specific antisera. Specific inhibitory effect of antibody on adsorptive activity of *M. pneumoniae* was tested with hyperimmune rabbit, guinea pig, and mule sera (Table 4). Hemadsorption or cell adsorption to *M. pneumoniae* was inhibited only by *M. pneumoniae* immune sera. Preimmunization serum or antisera prepared to other mycoplasmas failed to block such activity. Heat inactivation at 60 C for 30 min did not affect the adsorption inhibition activity of guinea pig or mule *M. pneumoniae* antiserum, whereas that of rabbit antiserum decreased fourfold after such treatment. However, when 5% fresh guinea pig serum, which was without effect upon hemadsorption, was added to heated rabbit antiserum, its original adsorption inhibition activity was restored. This suggests that a heat labile accessory factor was required for the action of rabbit adsorption-inhibiting antibody. RDE treatment did not affect adsorption inhibition potency of the *M. pneumoniae* antisera. When fractions of immune sera separated on Sephadex G-200 gel were examined, hemadsorption inhibition activity was found in both 19*S* and 7*S* immunoglobulins. The 7*S* antibodies predominated in guinea pig and mule antisera, whereas 19*S* antibodies predominated in rabbit antisera.

Hemadsorption inhibition blocking activity of M. pneumoniae and its fractions. To determine which components of *M. pneumoniae* were involved in adsorption, various fractions extracted from this organism were tested for their capacity to block adsorption-inhibiting antibody. In addition, the fractions were tested for complement-fixing activity and for their capacity to block metabolism-inhibiting antibody.

Results of these experiments are summarized in Tables 5, 6, and 7. Eight units of rabbit hemadsorption-inhibiting antibody were blocked by a suspension of *M. pneumoniae* (whether tested unheated or after incubation at 56 C for 30 min), by its lipid fraction, and by glycerophospholipid hapten of the lipid fraction (silicic acid column fraction 8). The other silicic acid column fractions of *M. pneumoniae* lipid, as well as the Sephadex chromatographic fractions, and the chemically extracted proteins and polysaccharides were negative. However, when the first Sephadex G-25 chromatographic fraction was split into its pro-

TABLE 4. *Effect of Mycoplasma antisera on adsorption by M. pneumoniae*

Serum	Treatment	Reciprocal of serum dilution-inhibiting adsorption	
		Guinea pig erythrocytes	Monkey tracheal epithelial cells
Mule			
Preimmunization	None	<1	<1
Postimmunization, *M. pneumoniae*	None	256	256
	60 C, 30 min or RDE[a]	256	256
Guinea pig			
Preimmunization	None	<1	<1
Postimmunization, *M. pneumoniae*	None	16	16
	60 C, 30 min or RDE	16	16
Rabbit			
Preimmunization	None	<1	<1
Postimmunization, *M. pneumoniae*	None or RDE	16[b]	16
	60 C, 30 min	4	4
	60 C, + 5% fresh guinea pig serum[c]	16	16
M. hominis, *M. orale* 1, *M. orale* 2, *M. salivarium*, *M. fermentans*, *M. gallisepticum*, or *M. pulmonis*	None	<1	<1

[a] Eight units of RDE.
[b] Same titers obtained when incubation was performed at 4 C, room temperature, or 37 C.
[c] Fresh guinea pig serum (5%), by itself, did not effect hemadsorption or cell adsorption.

TABLE 5. *Blocking of hemadsorption-inhibiting antibody by M. pneumoniae and its fractions*

M. pneumoniae immune serum	Hemadsorption-inhibiting antibody units blocked by indicated material											
	M. pneumoniae suspension[a] (2%)		Lipid extract (2%)	Silicic acid column chromatographic fractions of lipid extract		Protein 1, 2; polysaccharide 1, 2, 3 fractions (2%)	Lipoprotein complex (Sephadex G-25 fraction 1)			Sephadex G-25 fraction 2 (1%)	Sephadex G-25 fraction 3, 4 (1%)	*M. gallisepticum* or *M. fermentans* suspension[a] (2%)
	Unheated	56 C for 30 min		1-7, 9-10, (0.1%)	8,[b] glycerophospholipid hapten (0.1%)		Whole fraction (1%)	Lipid extract (1%)	Protein fraction (2%)			
Rabbit no. 1	8	8	8	0	8	0	0	4	0	0	0	0
Rabbit no. 2	8	8	8	0	8	0	0	NT	NT	0	0	0
Guinea pig	8	0	0	0	0	0	1[c]	0	0	0 or 1	0	0
Mule	8	0	0	0	0	0	8[d]	0	0	1 or 2	0	0

[a] Sonically treated, 10 kc for 30 min. Percentage values for each material tested indicate per cent by weight.

[b] Eluted with chloroform-methanol (9:1).

[c] Four units of hemadsorption-inhibiting antibody blocked when fraction used at 2% concentration.

[d] When heated (56 C for 30 min), the fraction lost its hemadsorption-inhibiting blocking activity.

TABLE 6. *Blocking of metabolism-inhibiting antibody by M. pneumoniae and its fractions*

M. pneumoniae immune serum	Reciprocal of the highest dilution of the indicated material which blocked 8 units of metabolism-inhibiting antibody										
	M. pneumoniae suspension[a] (2%)		Lipid extract (2%)	Glycerophospholipid hapten (0.1%)	Protein 1, 2 polysaccharide 1, 2, 3 fractions (2%)	Lipoprotein complex (Sephadex G-25 fraction 1)			Sephadex G-25 fraction 2 (1%)	Sephadex G-25 fraction 3, 4 (1%)	*M. fermentans* suspension[a] (2%)
	Unheated	56 C for 30 min				Whole fraction (1%)	Lipid extract (1%)	Protein fraction (2%)			
Rabbit no. 1	256	128	1,024	256	<1	10	80	<1	1	<1	<1
Rabbit no. 2	128	64	>1,024	64	<1	10	160	<1	1	<1	<1
Mule	256	2	4	8	<1	10	320	<1	1	<1	<1

[a] Sonically treated, 10 kc for 30 min. Percentage values for each material tested indicate per cent by weight.

tein and lipid components, the latter fraction also blocked hemadsorption-inhibiting activity of rabbit serum. The failure of the original Sephadex G-25 fraction 1 to block antibody probably reflects the fact that it contained only 15% lipid by weight. On a weight basis, glycerophospholipid hapten was the most potent hemadsorption inhibition blocking material.

In contrast, when guinea pig and mule sera were tested, *M. pneumoniae* and its fractions exhibited a different pattern of hemadsorption-inhibiting antibody blocking activity. Only the unheated suspension of *M. pneumoniae* or the lipoprotein Sephadex G-25 fraction 1 blocked hemadsorption-inhibiting antibodies in these sera and their immunoglobulins. The observations made with the rabbit, guinea pig, and mule antisera appeared to be specific, since a suspension of *M. gallisepticum* or *M. fermentans* failed to block hemadsorption-inhibiting activity of these *M. pneumoniae* antisera.

With one exception (Sephadex G-25 fraction 1 and mule antiserum), suspensions or fractions of *M. pneumoniae* which reacted with hemadsorption-inhibiting antibodies also reacted with metabolism-inhibiting and complement-fixing antibodies. However, a number of materials that did not react with hemadsorption-inhibiting antibodies were reactive when assayed by the complement fixation or metabolism-inhibiting antibody blocking technique. Fractions which were reactive in any of the assay systems always contained lipid.

TABLE 7. *Complement-fixing activity of M. pneumoniae and its fractions*

M. pneumoniae immune serum	Reciprocal of the highest dilution of the indicated material which reacted with 8 units of complement-fixing antibody										
	M. pneumoniae suspension[a] 2%		Lipid extract (2%)	Glycerophospholipid hapten (0.1%)[b]	Protein 1, 2 polysaccharide 1, 2, 3 fractions (2%)	Lipoprotein complex (Sephadex G-25 fraction 1)			Sephadex G-25 fraction 2 (1%)	Sephadex G-25 fraction 3, 4 (1%)	*M. gallisepticum* or *M. fermentans* suspension[a] (2%)
	Unheated	56 C for 30 min				Whole fraction (1%)	Lipid extract (1%)	Protein fraction (2%)			
Rabbit no. 1..	512	256	512	2,048	<4	512	640	<4	64	<4	<4
Rabbit no. 2..	256	64	256	1,024	<4	512	640	<4	256	<4	<4
Guinea pig...	1,024	128	512	256	<4	512	2,560	<4	256	<4	<4
Mule.....	1,024	128	128	512	<4	512	640	<4	256	<4	<4

[a] Sonically treated, 10 kc for 30 min. Percentage values for each material tested indicate per cent by weight.

[b] This silicic acid column fraction of the lipid extract, eluted with chloroform-methanol (9:1) exhibited a high level of complement-fixing activity with a serum from a patient convalescent from *M. pneumoniae* pneumonia; a 1 *p*65,536 dilution of the hapten (0.1%) fixed complement with this serum. The other nine silicic acid column fractions were without complement-fixing activity when tested with the convalescent serum.

TABLE 8. *Stimulation of hemadsorption-inhibiting antibody by M. pneumoniae and its fractions*

Material used for immunization (1% suspension)	Reciprocal of hemadsorption inhibition antibody titers of sera of animals immunized with indicated material	
	Rabbit	Guinea pig[a]
M. pneumoniae[b]	16	16
Chemically extracted fractions		
Lipid	<1	<1
Polysaccharide 1, 2, or 3	NI[c]	<1
Protein 1	NI	<1
Protein 2	<1	2
Chromatographic fractions (Sephadex G-25)		
Fraction 1	8	32
Fraction 2	2	<1
Fraction 3 or 4	NI	<1

[a] Each value represents the titer of pooled sera from two to three guinea pigs.

[b] Sonically treated, 10 kc for 30 min.

[c] Rabbits not immunized with these fractions.

Stimulation of hemadsorption inhibition antibody by M. pneumoniae fractions. Chemically extracted and chromatographic fractions of *M. pneumoniae* were tested for their capacity to stimulate production of hemadsorption-inhibiting antibodies in animals (Table 8). Besides the whole organism, only Sephadex G-25 fraction 1 stimulated significant levels of hemadsorption-inhibiting antibodies in rabbits and guinea pigs.

DISCUSSION

Virulence of *M. pneumoniae* for man has been well documented; however, the pathogenesis of disease still remains to be determined (3). It was suggested that the quantity of peroxide secreted by *M. pneumoniae* may contribute to its virulence (Sobeslavsky and Chanock, *in press*).

The results of this study provide additional information which may have relevance to the mechanism by which *M. pneumoniae* damages lung tissue. Previously it had been shown that this organism can adsorb erythrocytes (derived from several species), human spermatozoa, and human heteroploid tissue culture cells (17, 18; R. A. Del Giudice and R. Pavia, Bacteriol. Proc., p. 71, 1964). In this study, we found that the organism binds firmly to tracheal epithelial cells derived from monkeys, rats, and chickens. The affinity of *M. pneumoniae* for respiratory epithelium, unique among the mycoplasmas which infect man, may play a role in virulence because this type of attachment provides an unusual opportunity for peroxide secreted by the organism to attack the tissue cell membrane without being rapidly destroyed by catalase or peroxidase present in extracellular fluids of the body. Cohen and Somerson showed that 10^9 to 10^{10} viable *M. pneumoniae* produced a quantity of peroxide (0.2 to 2 μmoles per hr), comparable to that generated by acetylphenylhydrazine, which hemolyzed red

cells through a peroxide effect (5–7). The disruptive effect of peroxide upon the host cell could well be magnified if it acted directly upon the cell membrane rather than after diffusion through and dilution in extracellular fluids.

Although the adsorption reaction between *M. pneumoniae* and respiratory epithelial cells may explain, in part, the virulence of the organism, the experimental findings fail to explain the natural host specificity of *M. pneumoniae*, since the organism exhibited firm binding not only to primate tracheal epithelium but also to epithelial cells from the rat and the chicken trachea. Thus far, *M. pneumoniae* has not been recovered from rats or chickens under natural conditions. In contrast, under experimental conditions, both of these species can be infected with *M. pneumoniae* that localizes in the lower respiratory tract, producing pneumonitis in rats and a silent infection in chick embryos (8).

The red cell and tracheal epithelial cell receptor was apparently neuraminic acid, since it was removed by treatment of cells with RDE, purified neuraminidase, or influenza B virus. In addition, pretreatment of *M. pneumoniae* with neuraminic acid or neuraminelactose prevented adsorption of erythrocytes or respiratory tract cells. It is of interest that *M. gallisepticum*, which is a respiratory tract pathogen of birds, attaches to similar receptors on red cells (9) and on respiratory epithelial cells. The other mycoplasmas tested differed in either (i) failing to adsorb to cells or (ii) adsorbing to cells through non-RDE sensitive receptors.

The adsorption sites on *M. pneumoniae* were specifically blocked by homologous but not heterologous antisera. This property made it possible to study the nature of the mycoplasma adsorption sites by testing the capacity of different fractions of the organism to block the action of hemadsorption-inhibiting antibodies. Antibodies of this type in rabbit antisera were blocked by the lipid extract of *M. pneumoniae* and by the glycerophospholipid hapten of the organism but not by protein or polysaccharide fractions. This indicates that the glycerophospholipid hapten, which is the serologically active component primarily responsible for reaction with complement-fixing antibodies in all antisera and metabolism-inhibiting antibodies in human and rabbit antisera, was also involved in the adsorption reaction with tissue cells (11). A component of *M. pneumoniae* other than the glycerophospholipid hapten also appeared to be involved, since the hemadsorption inhibition activity of guinea pig and mule antisera was not blocked by the lipid extract of the organism nor by the hapten. Unlike the glycerophospholipid hapten, the other active component was not heat stable, since exposure of *M. pneumoniae* to 56 C for 30 min abolished its blocking activity for hemadsorption-inhibiting antibodies in guinea pig and mule sera. The nature of this additional component was not defined; however, the finding that Sephadex G-25 fraction 1 (rich in lipoprotein) inhibited hemadsorption-inhibiting antibodies in mule and guinea pig antisera suggested that the component may be lipoprotein or a constituent of lipoprotein.

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