Ultrastructural Lesions in *Mycoplasma pneumoniae* Membranes Produced by Antibody and Complement

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Lesions in *Mycoplasma pneumoniae* membranes were produced by antibody and either human or guinea pig complement and were observed by electron microscopy after negative staining. These defects measured approximately 9.0 to 10.0 nm in diameter—slightly more for human complement than for guinea pig complement. The development of these small lesions was accompanied by a decrease in the viability of the organism.

Immune cytotoxicity mediated by the complement system can be initiated by antibody bound to cell surface antigens. As the result of the action of antibody and complement, small lesions have been shown in membranes of a wide variety of cells including erythrocytes, Krebs ascites tumor cells, bacteria, virus particles, and artificial membranes (1, 3-5 11, 13).

In bacteria, the study of this reaction is hampered by the presence of the cell wall. *Mycoplasma* lack a rigid cell wall, thus exposing their membrane directly to the environment. *Mycoplasma pneumoniae* is a cause of atypical pneumonia in man and the only mycoplasma conclusively shown to produce disease in humans.

Negatively stained preparations of *M. pneumoniae* contain pleomorphic organisms which most often are filamentous in form (2). In a previous paper we described that *M. pneumoniae* organisms treated with antibody and complement lose their filamentous appearance and are transformed to ghost-like structures with large, irregular holes in their membranes (8).

In this report we show that ultrastructural lesions are 9.0 to 10.0 nm in diameter and are produced by antibody and complement acting on the membrane of *M. pneumoniae*.

MATERIALS AND METHODS

Organisms and culture conditions. The PI 1428 strain of *M. pneumoniae* was used in its 5th, the FH

strain in its 298th passage, on artificial medium. The organisms were grown in broth medium consisting of 7 parts PPLO broth (Difco Laboratories, Detroit, Mich.), 2 parts agamma horse serum (Baltimore Biological Laboratories, Cockeysville, Md.), 1 part 25% yeast extract (Flow Laboratories, Inc., Rockville, Md.), and 1% glucose (9). The pH of the medium was adjusted to 7.5 by 0.1 N sodium hydroxide. The organisms were grown on the glass surface of 5-liter Povitsky bottles containing 1 liter of medium (17). After 5 to 6 days of incubation at 37 C, the medium was decanted, and the sheet of organisms adhering to the glass surface of two bottles was scraped off into 10 ml of 0.01 M N-tris (hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES)-buffered saline containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂. This procedure yielded a highly concentrated organism suspension which was kept at 0 C until used. The optical density at 600 nm was about 0.5 for the PI 1428 strain and 0.9 for the FH strain. Agar medium was employed as described previously (7, 9).

Antiserum and complement. Rabbit hyperimmune serum against *M. pneumoniae* was prepared as described previously (19). This serum had a complement-fixing (CF) antibody titer of 1:512 tested by the microtiter technique (19). The metabolism-inhibiting (MI) antibody titer tested by inhibition of glucose utilization by the organisms was 1:4096 (18). The serum was heated at 56 C for 30 min before use. Fresh guinea pig serum (GPS) and fresh human serum (HS) served as the sources of complement. The preparation of GPS has been described in detail previously (7, 15). The GPS was shown to be free from detectable antibody to *M. pneumoniae*. More than 90% of adult human sera contain antibody to *M. pneumoniae* detectable by sensitive methods (H. Brunner, W. D. James, R. L.

Horswood, and R. M. Chanock, *unpublished data*). We, therefore, selected serum of an individual with a very low antibody titer as the complement source. The antibody titer measured by the mycoplasma-cidal test (MCT) was 1:157 (7).

Adsorption. To absorb out natural antibody, 1 ml of HS was exposed to 1 ml of viable *M. pneumoniae* organisms (approximately 10^9 colony-forming units/ml) for 2 hr at 0 C. Subsequently, the reaction mixture was centrifuged at $36,000 \times g$ for 30 min at 4 C and then filtered through a 220-nm pore size filter (Millipore Corp. Bedford, Mass.). No viable organisms could be detected in the HS after the described treatment. The MCT-antibody titer was 1:16 after the adsorption procedure, whereas CF or MI antibody were not detectable.

Reaction mixtures. One milliliter of the *M. pneumoniae* suspension was mixed with 0.5 ml of rabbit hyperimmune serum diluted 1:50 in TES-buffered saline with Ca^{2+} and Mg^{2+} . After incubation for 60 min at 4 C, either fresh HS or GPS was added. The complement preparations were not prediluted. Controls containing antibody alone, complement alone, or buffer alone were included. The reaction mixtures were incubated for 1 hr in a 37 C water bath and then diluted 1:10 in ice-cold TES-buffered saline without Ca^{2+} and Mg^{2+} to stop the reaction. Samples (0.1 ml) were removed for titration of viable organisms. The suspensions were then centrifuged at $36,000 \times g$ for 10 min at 4 C, the pellets were washed twice in 5-ml amounts of TES-buffered saline without additives and finally resuspended in 0.5 ml of buffer.

Electron microscopy. Drops of the resuspended pellets were immediately transferred onto 200-mesh Formvar-coated copper grids; after 30 sec excess fluid was removed with filter paper, and a drop of either 1% ammonium molybdate or 2% sodium phosphotungstate, which had been filtered through 220- and 50-nm membrane filters (Millipore Corp) and adjusted to pH 7.2, was added and blotted after approximately 30 sec. The grids were examined in a Siemens Elmiskop 1A at 80 kv with a 50- μm objective aperture (6, 8).

RESULTS

Decrease in viability of *M. pneumoniae* by antibody and human complement. As indicated in Table 1, a high concentration of antibody to *M. pneumoniae* caused a 10-fold decrease in viability of an unfiltered, concentrated organism suspension in the presence of fresh HS. Antibody alone and complement alone did not cause a significant killing effect under these conditions. This indicates that antibody alone neither killed nor aggregated

TABLE 1. Decrease in viable organisms and approximate proportion of ghosts of *M. pneumoniae* after incubation for 60 min at 37 C in indicated reaction mixture

Reaction Mixture	Log_{10} CFU/ml after 60 min at 37 C ^a	Approximate proportion of ghosts seen by EM (%) ^b
<i>M. pneumoniae</i> strain FH Pass. 298		
+ Antibody + C' (HS)	8.0	>10.0
+ Antibody	9.1	<1.0
+ C' (HS)	9.1	1.0
+ Antibody and C' (HS, 56 C, 30 min)	8.9	<1.0
+ Buffer	9.3	<1.0
<i>M. pneumoniae</i> strain PI 1468 Pass. 5		
+ Antibody + C' (HS)	6.5	>10.0
+ Antibody	7.4	<1.0
+ C' (HS)	7.7	1.0
+ Antibody + C' (HS, 56 C, 30 min)	7.3	<1.0
+ Buffer	7.9	<1.0

^a CFU, Colony-forming units.

^b EM, Electron microscopy

M. pneumoniae organisms under the experimental conditions. Heating of complement abolished the killing effect of antibody on *M. pneumoniae* organisms.

Ultrastructural lesions in *M. pneumoniae* membranes. The suspensions of *M. pneumoniae* used for electron microscopy contained many aggregates of highly entangled filaments, but a large number of single filamentous organisms were also observed. The surface membrane of untreated organisms was intact (Fig. 1). When specific antibody for *M. pneumoniae* and either GPS or HS were added, a large proportion of ghost-like organisms without filaments were seen, and these contained one or more ultrastructural lesions on their membranes (Fig. 2-4). The appearance of these "holes" was similar to that described for other biologic membranes exposed to antibody and complement. The "holes" contained a dark central portion which was surrounded by a single layered ring of increased density. The dark central portion was often irregular in outline. A few membranes had up to 200 holes on their surface. However, some organisms retained their filamentous appearance, and no lesions were observed in

FIG. 1. *M. pneumoniae* incubated for 60 min at 4 C and for 60 min at 37 C in buffer and negative-stained with 1% ammonium molybdate. Filamentous electron-dense organism with a smooth membrane can be seen. $\times 118,200$.

FIG. 2. *M. pneumoniae* incubated for 60 min at 4 C with antibody, subsequently incubated for 60 min at 37 C with HS, and negatively stained with 1% ammonium molybdate. A ghost membrane with multiple small "holes" is shown. $\times 157,600$.

these healthy-looking organisms. In preparations containing fresh HS alone, a small proportion of organisms were lysed, and a few lesions were visible on the cell surfaces, but the addition of rabbit *M. pneumoniae* hyperimmune serum increased the number of lysed cells and the number of lesions many-fold. Sometimes the lesions were confluent and clusters of holes could be observed. The diameter of these holes was 9.0 to 10.0 nm for HS and 9.0 to 9.5 nm for GPS.

Material was seen adhering to the surface of the organisms; this was probably antibody or complement, or both, although other material in the antiserum or HS might be involved (Fig. 2-4). The existence of protrusions described for *M. gallisepticum* by Chu and Horne (10) cannot explain this adhering material because it was not present on the normal *M. pneumoniae* membrane (Fig. 1).

Discussion

The mycoplasma membrane has been the subject of intensive biochemical and morphological studies because mycoplasma lack a rigid cell wall (16). They are, furthermore, the smallest organisms known which can be cultivated on artificial medium. These distinct properties led us to study the question whether lesions could be produced in the membranes of this group of microorganisms by antibody and complement.

The results indicate that ultrastructural lesions in the limiting membrane of *M. pneumoniae* are visible after the combined action of specific antibody and complement. These holes can be seen in membrane pieces of different size, and resemble in their morphological properties those in other biological membranes which have been similarly treated (14).

Naturally occurring antibody to bacteria in fresh human sera have been shown to be sufficient for the production of lesions (3). In a study in progress we found that a very high proportion of adults had antibody in their sera when sensitive serological techniques were used. We were, therefore, not surprised that the fresh human serum which we used as the source of complement contained antibody to *M. pneumoniae* and produced lesions in the organism's membrane without addition of

antiserum. The number of ghosts and of lesions was, on the other hand, greatly increased by the addition of specific antiserum. The antibody in HS could not be completely absorbed by living or heat killed *M. pneumoniae* organisms without losing an appreciable amount of complement activity.

The findings that these pits can be produced in different types of membranes suggests that a common mechanism is probably involved. Humphrey and Dourmashkin suggested, based on observations on sheep red blood cell membranes, that lipids might be involved (13). Our recent finding that specific antibody to membrane glycolipids of *M. pneumoniae* can sensitize the organism to the lytic action of complement provides support for this view (8).

Although our results add nothing to the understanding of the mechanisms of this reaction, mycoplasma membranes may prove to be a useful model for further investigations on the action of complement on antibody-sensitized membranes of nucleated cells because no physical or chemical treatment is necessary to remove a cell wall. In addition, the growth of mycoplasma on glass makes it possible to obtain a large quantity of concentrated organisms in a relatively pure form without prior physical stress such as that exerted by centrifugation (17).

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LITERATURE CITED

1. Berry, D. M., and J. D. Almeida. 1968. The morphological and biological effects of various antisera on avian infectious bronchitis virus. *J. Gen. Virol.* **3**: 97-102.
2. Biberfeld, G., and P. Biberfeld. 1970. Ultrastructural features of *Mycoplasma pneumoniae*. *J. Bacteriol.* **102**:855-861.
3. Bladen, H. A., R. T. Evans, and S. E. Mergenhagen. 1966. Lesions in *Escherichia coli* membranes after action of antibody and complement. *J. Bacteriol.* **91**:2377-2381.
4. Bladen, H. A., H. Gewurz, and S. E. Mergenhagen. 1967. Interactions of the complement system with the surface and endotoxin lipopolysaccharide of *Veillonella alcalescens*. *J. Exp. Med.* **125**:767-786.
5. Borsos, T., R. R. Dourmashkin, and J. H. Humphrey. 1964. Lesions in erythrocyte membranes caused by

FIG. 3. A portion of the membrane of Fig. 2 is shown at a higher magnification to provide a better comparison with Fig. 4. $\times 250,000$.

FIG. 4. *M. pneumoniae* incubated for 60 min at 4 C in buffer containing 1:50 antiserum to *M. pneumoniae* and then for 60 minutes at 37 C after addition of HS, negatively stained with 2% sodium phosphotungstate. Small distinct lesions can be seen. $\times 250,000$.

- immune hemolysis. *Nature (London)* **202**: 251-252.
6. Brenner, S., and R. W. Horne. 1959. A negative staining method for high resolution electron microscopy of viruses. *Biochim. Biophys. Acta* **34**:103-110.
 7. Brunner, H., W. D. James, R. L. Horswood, and R. M. Chanock. 1972. Measurement of *Mycoplasma pneumoniae* mycoplasmacidal antibody in human serum. *J. Immunol.* **108**:1491-1498.
 8. Brunner, H., S. Razin, A. R. Kalica, and R. M. Chanock. 1971. Lysis and death of *Mycoplasma pneumoniae* by antibody and complement. *J. Immunol.* **106**:907-915.
 9. Chanock, R. M., L. Hayflick, and M. F. Barile. 1962. Growth on artificial medium of an agent associated with atypical pneumoniae and its identification as a PPLO. *Proc. Nat. Acad. Sci. U.S.A.* **48**:41-49.
 10. Chu, H. P. and R. W. Horne. 1967. Electron microscopy of *Mycoplasma gallisepticum* and *Mycoplasma mycoides* using the negative staining technique and their comparison with *Myxovirus*. *Ann. N. Y. Acad. Sci.* **143**:190-203.
 11. Glynn, A. A., and C. M. Milne. 1967. A kinetic study of the bacteriolytic and bactericidal action of human serum. *Immunology* **12**:639-653.
 12. Haxby, J. A., C. B. Kinsky, and S. C. Kinsky. 1968. Immune response of a liposomal model. *Proc. Nat. Acad. Sci. U.S.A.* **61**:300-307.
 13. Humphrey, J. H., and R. R. Dourmashkin. 1965. Electron microscope studies of immune cell lysis, p. 179-189. *In* G. E. W. Wolstenholme and J. Knight (ed.), *Complement*. Little, Brown, and Company, Boston.
 14. Humphrey, J. H., and R. R. Dourmashkin. 1969. The lesions in cell membranes caused by complement. *Advan. Immunol.* **11**:75-115.
 15. Mayer, M. M. 1961. Complement and complement fixation, p. 133-240. *In* E. A. Kabat and M. M. Mayer, (ed.), *Experimental immunochemistry*. Charles C Thomas, Springfield.
 16. Razin, S. 1969. The *Mycoplasma* membrane, p. 317-348. *In* L. Hayflick (ed.), *The mycoplasmatales and the L-phase of bacteria*. Appleton-Century-Crofts, New York.
 17. Somerson, N. L., W. D. James, B. E. Walls, and R. M. Chanock. 1967. Growth of *Mycoplasma pneumoniae* on a glass surface. *Ann. N. Y. Acad. Sci.* **143**:384-389.
 18. Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock. 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg.* **64**:91-104.
 19. Taylor-Robinson, D., N. L. Somerson, H. C. Turner, and R. M. Chanock. 1963. Serological relationships among human mycoplasmas as shown by complement fixation and gel diffusion. *J. Bacteriol.* **85**:1261-1273.