Ultrastructure of Mycoplasmal Capsules as Revealed by Stabilization with Antiserum and Staining with Ruthenium Red

Masanori TAJIMA, Takeshi YAGIHASHI, and Tetsuo NUNOYA

Nippon Institute for Biological Science, 2221-1 Shin-machi, Ome, Tokyo 198, Japan

(Received 15 November 1984/Accepted 25 December 1984)

ABSTRACT. The collapse of the capsular glycocalyx was effectively prevented, when tissue blocks from the pneumatic lungs of pigs infected with Mycoplasma hyopneumoniae or the tracheas of chickens infected with M. gallisepticum were treated with respective specific antisera before fixation and staining with ruthenium red. The antisera prepared against different mycoplasma species and preimmune sera had no stabilizing effects on the capsular glycocalyces. The stabilized capsules of M. hyopneumoniae extended for approximately 125 nm outside the limiting membrane and were 3 times thicker and less dense than those of cells not treated with specific antiserum or cells exposed to heterologous antiserum or preimmune serum. The thickness of the stabilized capsules of M. gallisepticum measured about 40 nm, whereas that of untreated capsules was about 20 nm. In both mycoplasma species, the capsules appeared to consist of fibrillar elements, seemingly being radially oriented, and fine granular material. The capsular material extended to spaces separating mycoplasmal cells from each other and from the epithelial surface, and seemed to play an important role in the colonization of the respiratory tract of pigs or chickens. In the case of M. gallisepticum, however, the terminal bleb was considered to be the primary attachment factor.—KEY WORDS: Electron microscopy, Mycoplasma hyopneumoniae, M. gallisepticum, capsule.


A variety of mycoplasma species have been shown to possess a capsule that could be visualized by electron microscopy following staining with ruthenium red. The capsule was associated with cytadsorption [13, 24, 27], virulence [11, 14, 22, 24] or hemagglutinating properties [2] of mycoplasmas.

Bayer and Thurow [3], in a study on the polysaccharide capsule of Escherichia coli, observed that dehydration for electron microscopic preparations in ≥50% acetone or alcohol caused the capsule to collapse into thick bundles and this was prevented by pretreatment of the cell with capsule-specific immunoglobulin G. A similar stabilizing effect of specific antibody on the capsular polysaccharide has been demonstrated in group B streptococci [15]. More recently, Chan et al. [4] applied anti-K30 antibody to ileal tissues from calves infected with enterotoxigenic E. coli before staining with ruthenium red, and demonstrated bacterial microcolonies surrounded by an uncollapsed, thick glycocalyx and in close contact with the epithelial cell surface. These studies prompted us to use this technique to elucidate the morphologic properties of capsules of Mycoplasma hyopneumoniae and M. gallisepticum and the role of capsular material in the interaction of both mycoplasma species with their host cells. This report describes the results obtained by these observations.

MATERIALS AND METHODS

Mycoplasma strains and animals: Two species of swine mycoplasmas and one strain of M. gallisepticum were used. Pig-passaged strain MI-3 of M. hyopneumoniae was used at passage 10 [22]. The strain BTS-7 of M. hyorhinis was originally isolated by Switzer [20] and provided by Dr. K. Yamamoto, University of Tokyo, Japan. M. gallisepticum strain SAS was isolated at the authors'
laboratory [21].
Specific-pathogen-free miniature pigs of the Pitman-Moore strain, 65-day-old, and White Leghorn chickens of line S, 50-day-old, known to be free of mycoplasmas were obtained from the Nippon Institute for Biological Science, Laboratory Animal Research Station, Kobuchizawa, Yamanashi Prefecture, and used throughout the study.

*Inoculation of pigs and chickens:* Two pigs were intranasally inoculated with 5 ml of a lung suspension from a pig infected with strain MI-3, and the dose was repeated 3 times at 2-day intervals. The inoculum was prepared as previously described [22] and contained $10^5$ color-changing units per ml. Two chickens were intratracheally inoculated with 0.5 ml of a suspension of strain SAS containing $10^5$ colony-forming units per ml.

*Preparation of antisera:* Suspensions containing approximately $10^{10}$ cells per ml of either *M. hyopneumoniae*, *M. hyorhinis* or *M. gallisepticum* were prepared. Each suspension was mixed with an equal volume of Freund's complete adjuvant and inoculated both subcutaneously and intramuscularly into an adult JW-NIBS rabbit with 0.5, 1.0, and 1.0 ml amount each at 3-day intervals. Two weeks after the last inoculation, each rabbit was given intravenously 1.0, 2.0 and 3.0 ml of the suspension at 3-day intervals. Blood was obtained from the rabbits 7 days after the last inoculation. The widths of inhibitory zones of the undiluted antisera, as determined by the disk growth inhibition test [7], were 4 mm for *M. hyopneumoniae*, 4 mm for *M. hyorhinis*, and 5 mm for *M. gallisepticum*.

*Procedures for examination:* The inoculated pigs and chickens were killed 3 and 2 weeks after the inoculation, respectively. The pig lungs and chicken tracheas were then subjected to examination by transmission electron microscopy (TEM). The procedures for fixation in the presence or absence of ruthenium red and embedding of these tissues for TEM have been described [21–23]. Specimens which were to be treated with antisera were immersed in either antiserum diluted 1:3 in phosphate-buffered saline for 1 hr at room temperature with frequent agitation prior to fixation. Thin sections were cut by using glass knives on a Porter-Blum ultramicrotome and stained with uranyl acetate followed by lead citrate. They were examined with a JEM-100B electron microscope, using an accelerating voltage of 80 kV.

**RESULTS**

*M. hyopneumoniae:* Mycoplasmas were seen in large numbers in the lumina of the respiratory tracts of the grossly affected lung lobes of 2 pigs. In lung tissue fixed in the presence of ruthenium red, a dark staining capsule, about 40 nm in thickness, was clearly seen external to the limiting membrane of the mycoplasmal cells. When tissue was treated with antiserum against *M. hyopneumoniae* before staining with ruthenium red, the capsule extended for approximately 125 nm outside the limiting membrane (Fig. 1). The antiserum-treated capsules appeared less compact with more marked surface irregularities and were stained less intensely than those of untreated cells, suggesting that the condensation of capsular polysaccharide occurring during dehydration for TEM preparation might be prevented by the application of specific antiserum. When viewed at higher magnification, the capsule appeared to consist of fine granular and fibrillar elements radiating from the outer layer of the unit membrane of the cells (Fig. 1, inset).

Most of mycoplasmas were located between the cilia and on the microvillous tips of the epithelial cells. The capsular material appeared to connect mycoplasmas with each other and to the ciliary and microvillous surface, enabling the organisms to adhere and to colonize to the mucosal surface of the respiratory tract (Fig. 2).

To exclude the possibility that a thickening
of the capsule seen in mycoplasmas treated with specific antiserum might be caused by
the nonspecific adsorption of proteins, infected lung tissues were treated with antiserum
prepared against *M. hyorhinis* or preimmune serum. The treatment with these sera did not
lead to any stabilization of capsular glyco-
calyx, and the capsules of cells thus treated
were indistinguishable, in thickness and appe-
airence, from those of untreated cells (Fig.
3).

*M. gallisepticum:* Mycoplasmas were
readily detected in the tracheal lumen, mostly
in close apposition to the luminal surface. In
mycoplasmas stained with ruthenium red fol-
lowing stabilization with specific antiserum,
the capsule appeared to be a dark layer ex-
tending about 40 nm from the cell surface,
and its fine structure was similar to that of *M.
hyopneumoniae* described above (Fig. 4).
The thickness of the capsule of mycoplasmas
not treated with antiserum measured approxi-
mately 20 nm and it was stained more inten-
sely than that of antiserum-treated organ-
isms (Fig. 5). Most of mycoplasmas attached
to the epithelial cells by their terminal blebs
close to the host cell membrane. In thin
sections of the tracheal tissues exposed to
antiserum, the capsular material frequently
appeared to bridge the interspace between
membrane region, other than that of the ter-

minal bleb, and the plasma membrane of
adjacent microvilli or cilia, suggesting that
the capsule might be an additional means of
holding the mycoplasmas close to the host
cells (Fig. 4).
Fig. 2. A portion of the bronchiolar epithelium from the same pig as shown in Fig. 1. The tissue and section were processed as described for Fig. 1. The capsular material appears to interconnect the mycoplasmal cells and extend to the ciliary and microvillous surface. ×20,000.

Fig. 3. A portion of the bronchiolar epithelium of a pig treated as described for Fig. 1. The tissue was treated with heterologous antiserum and fixed in the presence of ruthenium red. The section was stained as described in Fig. 1. The capsular material has collapsed, forming a compact and electron-dense layer. ×40,000.

Fig. 4. A portion of the tracheal epithelium of a chicken inoculated intratracheally with *M. gallisepticum* strain SAS and killed 2 weeks after inoculation. The tissue and section were processed as described in Fig. 1. The capsular material extending about 40 nm beyond the mycoplasmal limiting membrane appears to connect mycoplasmas with each other and to ciliary and microvillous surfaces. ×40,000.
It has been well documented that bacterial capsules are highly hydrated, containing as much as 98% water, and dehydration for microscopic preparations causes capsules to shrink to a small fraction of their original size [26]. In ultrastructural studies on *E. coli* [3, 4] and group B streptococci [15], it has been reported that the collapse of the bacterial glycocalyxes occurring during the dehydration steps could be prevented by crosslinking of the polysaccharide molecules with specific antibodies against capsular antigens. The mycoplasmal capsules demonstrable by ruthenium red staining are similar in appearance to those described in *E. coli* and streptococci and seem rich in water content. It is, therefore, probable that the capsules demonstrated by previous workers [2, 14, 22, 23] in several mycoplasmal species might not be manifested in their true dimensions and structure.

In the present study, antiserum prepared against whole cells of *M. hyopneumoniae* or *M. gallisepticum* was used instead of specific antibodies to capsular polysaccharides that had been employed in the case of bacteria. Nevertheless, the stabilization of capsular glycocalyx could be obtained by using homologous antiserum. The heterologous antiserum and preimmune serum failed in preventing the collapse of the capsular glycocalyx, indicating the specificity of the reaction.

In *M. hyopneumoniae* treated with homologous antiserum, the organisms were surrounded by a glycocalyx about 125 nm in thickness. The capsular material extended between the mycoplasma and neighboring microvilli or cilia and also interconnected the mycoplasmal cells. It thus appeared that the capsular glycocalyx plays an important role in the colonization of the respiratory tract by this mycoplasmal species.

Our results confirmed a widely accepted view that *M. gallisepticum* is an extracellular parasite which attaches to the epithelial cell surface by its terminal bleb [1, 6, 21, 25, 28]. In antiserum-treated organisms, the capsular material extending about 40 nm beyond the limiting membrane was also in close contact with the microvilli or cilia. These findings suggest that although the terminal bleb is the primary mechanism of cytadsorption, capsular glycocalyx might facilitate mycoplasmal adhesion to the epithelial surface. Such a presumption is supported by the observation that nonpathogenic strain KP-13 of *M. gallisepticum* had the terminal bleb but did not have a definite capsule, and when inoculated intratracheally into chickens, the organisms grew poorly and were gradually eliminated.
from the trachea, probably because of the lack of the adhesive properties [23].

The size of capsules has been correlated with virulence in several bacterial and mycoplasmal species [11, 16, 18, 22]. In *M. pulmonis*, a correlation was demonstrated between the cytodorsorption, pathogenicity for mice, and thickness of the capsule [24]. The bacterial capsules have been involved in such functions as adhesion of bacteria to surfaces [5, 9], protecting bacteria from surfactants [12], complement and antibodies [10, 17], phagocytes [8, 19], and attacking by bacteriophages [26]. The functions, other than cytodorsorption, of the mycoplasmal capsular glyco-calyx remain to be elucidated by further studies.

ACKNOWLEDGMENTS. This work was supported in part by a Grant-in-Aid for Scientific Research No. 58360030 from the Ministry of Education, Science and Culture of Japan.

REFERENCES


ULTRASTRUCTURE OF MYCOPLASMA CAPSULE


要約

抗血清による安定化及びルテニウム赤染色により示されるマイコプラズマ荚膜の微細構造: 田島正典・八木橋武・布谷鉄夫（日本生物科学研究所）—Mycoplasma hyopneumoniae 感染豚の肺及び M. gallisepticum 感染鶏の気管の組織を、それぞれの抗血清で処理した後、固定し、ルテニウム赤で染色することにより、マイコプラズマ（M）の荚膜糖衣の濃縮・崩壊が効果的に防止された。他種のMに対する抗血清と免疫前血清は荚膜安定効果をもたなかった。M. hyopneumoniae の安定化荚膜の厚さは約 125 nm で、非安定化荚膜に比し 3 倍厚く、かつ密度が低かった。M. gallisepticum の安定化荚膜は約 40 nm、非安定化荚膜は約 20 nm の厚さをもっていた。両種のMの荚膜は放射状に配列する微細纖維及び微細顆粒からなるようにみえた。荚膜物質はMと上皮表面との間の空隙を埋め、豚と鶏の呼吸路におけるMの定着において重要な役割を演じているように思われた。しかしながら M. gallisepticum の場合には、端末構造が主要な付着因子であると考えられた。