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Scanning Electron Microscopy of Mycoplasmas Adhering to Erythrocytes

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The interaction of *Mycoplasma pneumoniae* and *Mycoplasma gallisepticum* with human erythrocytes (RBC) was studied by scanning electron microscopy. The tight nature of the attachment of the microorganisms to the RBC was indicated by the indentation of the RBC surface at the site of attachment of *M. gallisepticum* cells and by traction and resulting distortion in the shape of the RBC at the point of its attachment to *M. pneumoniae* filaments growing on glass or plastic. In many cases attachment took place via the tip of the filaments, the membrane of the parasite appearing to be fused with that of the RBC. The morphology of the mycoplasmas growing on cover slips conformed in general with previous descriptions obtained by scanning electron microscopy. Growth of *M. pneumoniae* on glass or plastic consisted of branching filaments spread on the inert surface and microcolonies made up of intertwining filaments projecting into the medium. The filaments had a bulbous swelling adjacent to a tapered tip end. A few filaments were shown to have a ropelike helical twist. *M. gallisepticum* grown on the cover slips of Leighton tubes had a peculiar fusiform or teardrop shape with blebs at one or both poles of the cells. Elongated filamentous forms and chains of coccobacillary bodies were observed as well.

Adherence of the human pathogen *Mycoplasma pneumoniae* and the avian pathogen *Mycoplasma gallisepticum* to the respiratory epithelium appears to be a prerequisite for development of the respiratory disease caused by these parasites (1, 10, 23, 27). In an effort to chemically define the receptors on the host cells and the binding sites on the mycoplasmas responsible for adherence, we have developed several experimental systems based on adherence of mycoplasmas in suspension to erythrocytes (RBC) (3) and on adherence of RBC to sheets of mycoplasma growth on glass or plastic (11, 12, 14). Some information on the receptors, binding sites, and the factors influencing adherence has been reported in our previous communications (3, 11, M. Banai, S. Razin, W. Bredt, and I. Kehane, Infect. Immun., in press). The present communication deals with morphological aspects of mycoplasma adherence. The scanning electron microscope permits the visualization of large surface areas at relatively high resolution and thus appears to be particularly suitable for studying the adherence phenomenon. The electron micrographs shown here demonstrate the extremely tight nature of the attachment between the mycoplasmas and human RBC and also provide some additional information on the morphology of the mycoplasmas themselves.

MATERIALS AND METHODS

Organisms and growth conditions. *M. pneumoniae* strain FH was grown in Hayflick medium (15) containing 10% horse serum and 0.2% glucose. Growth was carried out either in Roux bottles containing 70 ml of medium and incubated horizontally or on glass cover slips submerged in petri dishes containing 5 to 10 ml of medium or in Leighton tubes equipped with a plastic cover slip (3393; Costar, Cambridge, Mass.) as described by Gallagher and Rhoades (13). The cultures were usually incubated for 48 h at 37°C, at which time the pH of the medium decreased to about 7.0. *M. gallisepticum* (A5969) was grown in a modified Edward medium (25) containing 4% horse serum. Growth was carried out in Erlenmeyer flasks or in Leighton tubes for 18 to 20 h at 37°C. In some experiments the mycoplasmas were labeled by supplementing the growth medium with 150 μCi of [9,10-^{3}H]palmitic acid (The Radiochemical Center, Amersham, England) per liter.

Mycoplasma suspensions. The sheet of *M. pneumoniae* growth on the surface of Roux bottles was washed three times with 10 ml of either phosphate-buffered saline (PBS), pH 7.2, or with 0.1 M NaCl containing 1 mM $CaCl_2$ and 0.05 M tris(hydroxymethyl)aminomethane, pH 7.2 (buffer A, reference 3). The organisms were then scraped off the glass with a rubber policeman into 4 ml of buffer. To remove or disaggregate the colonies in the scraped material, the suspensions were diluted to about 0.5 mg of cell protein per ml and filtered through an 8-μm membrane filter

(Millipore Corp., Bedford, Mass.) or a fiberglass filter (GF/c; Whatman Ltd., Clifton, N.J.). Another portion of the suspensions was sonicated in the cold in a sonicator (model W350; Heat Systems Ultrasonics, Inc., Plainview, N.Y.) with the small tip, 50% pulsed at an output of 1.5 for 40 s.

M. gallisepticum suspensions were prepared from 18- to 20-h broth cultures (absorbance of 0.15 to 0.20 at 640 nm). The organisms were harvested by centrifugation at 12,000 × *g* for 15 min, washed once in PBS, and suspended in PBS to a final cell concentration of 250 μg of protein per ml.

Trypsin treatment. *M. pneumoniae* organisms grown on glass cover slips were washed three times with buffer A and were then treated with 25 μg of trypsin (diphenyl carbamyl chloride-treated; Sigma Chemical Co., St. Louis, Mo.) per ml for 10 min at 37°C in the same buffer. Digestion was stopped by transferring the cover slips to a solution of 50 μg of the trypsin inhibitor *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) per ml in buffer A for 3 min at room temperature.

RBC. Fresh human RBC were washed 3 times in PBS or buffer A. The washed RBC pellet was suspended in the same buffer to give a 2 or 3% (vol/vol) packed-cell suspension (3).

Adherence of mycoplasmas to RBC. An attachment mixture consisting of 0.05 ml of a 2% RBC suspension and 0.1 ml of the labeled mycoplasma cell suspension in buffer A or in PBS (containing 15 to 25 μg of cell protein) was incubated in a siliconized test tube for 30 min at 37°C. The RBC with the attached mycoplasmas were separated by centrifugation and washed as described in detail by Banai et al. (3). Samples were taken for radioactivity measurements and for examination by scanning electron microscopy (SEM).

Adherence of RBC to mycoplasmas. Glass cover slips or the Leighton plastic cover slips containing *M. pneumoniae* or *M. gallisepticum* attached during growth were washed three times with PBS. A suspension of 3% RBC in PBS was layered over the growth surface of the cover slips. After incubation for 45 min at 37°C the cover slips were gently washed with PBS by transferring them three times through containers with PBS. The cover slips were then taken for SEM examination.

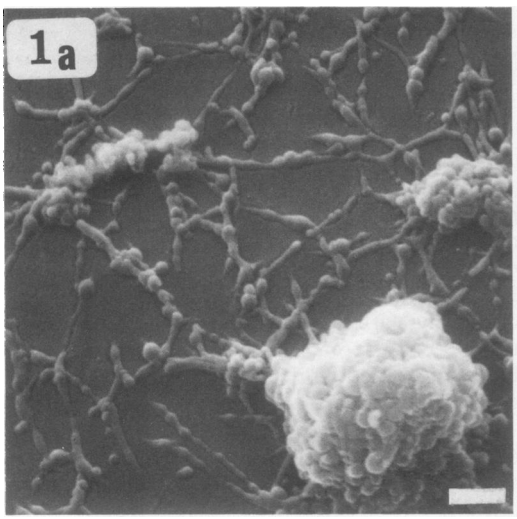
Preparation for SEM. Fixation of samples on cover slips was done by immersion in 1% glutaraldehyde in buffer A or in PBS or by adding glutaraldehyde directly to the culture medium to a final concentration of 1%. The samples were incubated with the fixative for 1 h at room temperature and then for 24 h at 4°C. Fixation of mycoplasmas in suspension with or without RBC was done by incubation with 1% glutaraldehyde for 1 h at room temperature. The suspensions were then layered over glass cover slips pretreated with 0.1% poly-L-lysine (20) for 1 h at room temperature, and then for an additional 24 h at 4°C. After fixation, samples were rinsed twice in buffer, dehydrated in a graded series of ethanol followed by ethanol-Freon 113, and critical point dried by using Freon 13 (9). The loaded cover slips were then stuck onto stubs using double sided sticky tape and sputter coated with a thin layer (10 to 20 nm) of gold palladium. Coated samples were examined in a Jeol 35 SEM equipped with a LaB_6 gun at an accelerating voltage of 35 kV. Micrographs were recorded on Polaroid type 55 positive/negative film.

RESULTS

***M. pneumoniae* morphology and attachment to RBC.** The filamentous shape was the dominating morphological feature of *M. pneumoniae* growing on glass cover slips and in Leighton tubes. Single or branching filamentous organisms with a bulblike "neck" and a tapered tip end were spread on the glass or plastic surface and formed an irregular meshwork (Fig. 1a and Fig. 2a and b). Microcolonies at various stages of development projected into the medium. The colonies appeared to consist of intertwining filaments. The ends of the filaments forming the colonies were usually rounded, so that superficially the colonies appeared to consist of spherical organisms (Fig. 1c). Trypsin treatment did not cause any significant detachment of organisms from the glass surface, but the cells appeared somewhat swollen and irregular in size (Fig. 3). Peculiar ropelike twisted filaments were seen in a few of our *M. pneumoniae* preparations (Fig. 4).

M. pneumoniae suspensions prepared by scraping off the growth from the surface of Roux bottles consisted mostly of microcolonies (Fig. 5a). Gentle sonication of the suspension resulted in partial disaggregation of the colonies (Fig. 5b), but at high magnification (not shown) many of the cells appeared to be swollen and were accompanied by minute fragments and debris. Filtration appeared to be more effective as a means of obtaining suspensions devoid of microcolonies, but over 85% of the organisms were retained on the filter (Fig. 5c and d).

The highest values for *M. pneumoniae* attachment to human RBC were obtained with untreated suspensions, whereas filtration and particularly sonication of the suspensions significantly decreased the attachment values (Table 1). SEM of untreated *M. pneumoniae* suspensions incubated with RBC showed the RBC to attach avidly to the mycoplasma microcolonies. The shape of the attached RBC appears to be somewhat distorted, as compared with that of unadsorbed RBC (Fig. 6). Electron microscopy of RBC adhering to *M. pneumoniae* growing on the plastic cover slip of Leighton tubes provided the most effective means for studying the interaction of the RBC with *M. pneumoniae*. The series of electron micrographs shown in Fig. 7 demonstrates that the attachment of an RBC to even a single filament is tight enough to produce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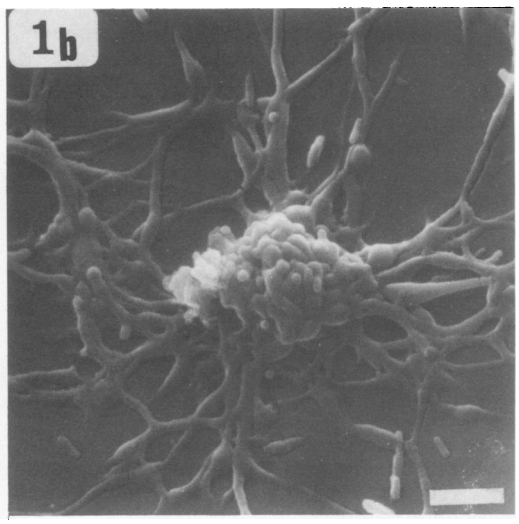
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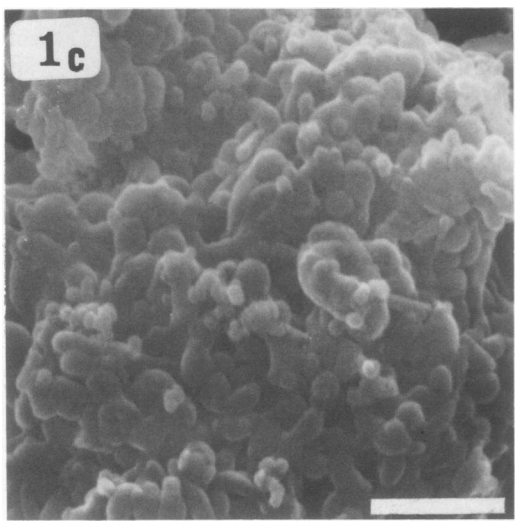
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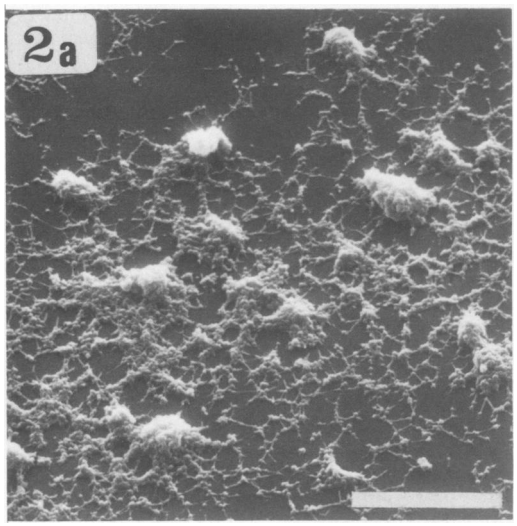
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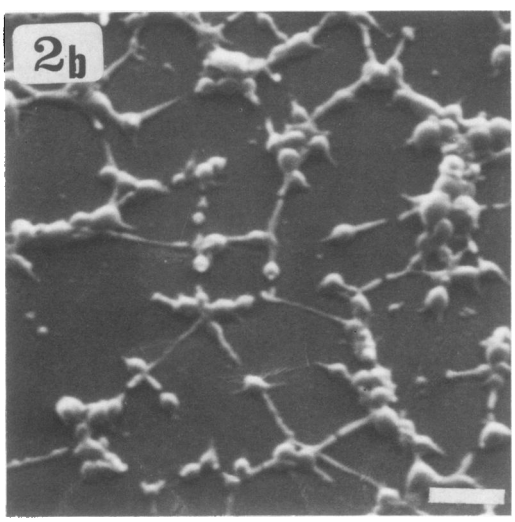
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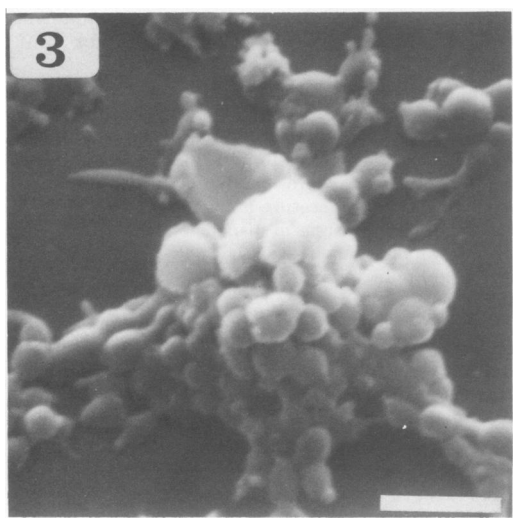
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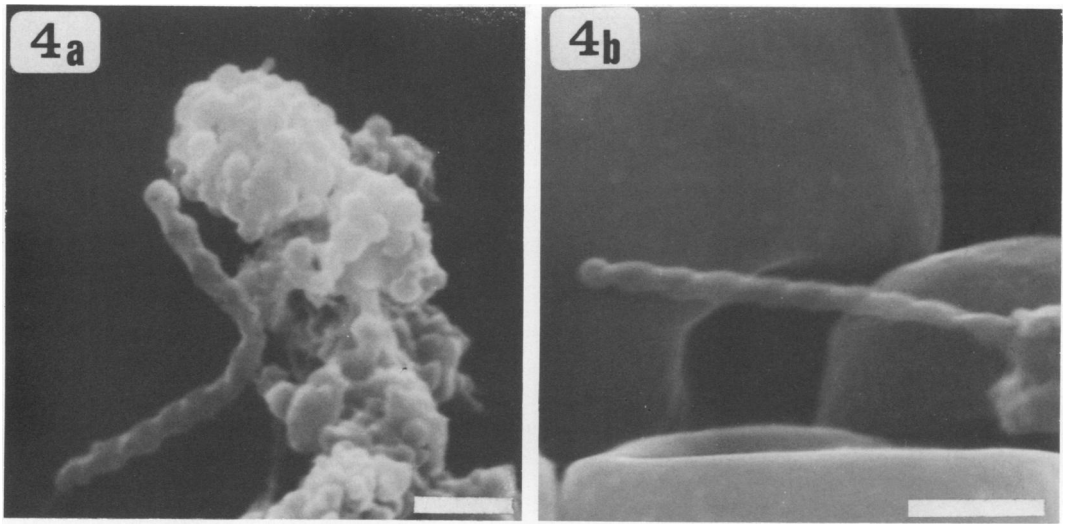

FIG. 4. *(a) Filament showing a rope-like helical twist adjacent to a microcolony of M. pneumoniae scraped off the glass surface. (b) A similar twisted filament of M. pneumoniae adjacent to RBC. Bar, 1 μm.*

considerable traction and distortion of the shape of the RBC. Furthermore, it appears that attachment can be affected via the tip of the filament (Fig. 7b and c). The possibility of fusion at the site of contact is suggested in Fig. 7d and e. Only RBC attached to mycoplasma filaments were distorted; unattached RBC preserved their characteristic bi-concave shape. The mycoplasma filaments stuck tightly to the plastic surface so that the RBC which attached to them could not be removed by washing. The physical stress imposed on the attached RBC by the washing fluid resulted in traction and shape distortion. The fixation step which followed washing apparently preserved the distorted shape.

***M. gallisepticum* morphology and attachment to RBC.** The peculiar fusiform or teardrop shape of *M. gallisepticum* could be demonstrated by SEM of organisms that became attached to the plastic cover slip during growth in Leighton tubes. The characteristic terminal bud-shaped swellings could be seen in many cells (Fig. 8). Short filamentous cells and chains of coccoid bodies characterize the growth of *M. gallisepticum* on the plastic surface. As estimated by radioactivity measurements, about 8% of the *M. gallisepticum* cells attached to the human RBC (Table 1). The tight nature of this attachment is suggested by the indentations of the RBC surface at the site of contact with the mycoplasmas (Fig. 9).

DISCUSSION

The morphological features of *M. pneumoniae* and *M. gallisepticum* revealed in our study conform, in general, with those reported by others (4, 5, 13, 18, 21, 22). Our preparations show the characteristic bulblike neck and tapered tip of the elongated or filamentous cells of *M. pneumoniae* growing on glass or plastic. These features were first observed by phase-contrast microscopy (6) and were later defined more clearly by SEM (4). The colonies of *M. pneumoniae*, which develop as outgrowths of the network of

FIG. 1. *Growth of M. pneumoniae on the plastic cover slip of a Leighton tube, 48-h culture fixed with 1% glutaraldehyde in situ. (a) General view showing the filamentous organisms attached to the plastic and microcolonies at various stages of development. The bulbous swellings and tapered tip ends can be seen on the filaments spread on the plastic surface. (b) Microcolony consisting of intertwined branching filaments. (c) Higher magnification of a microcolony. It is clear that the microcolony is made up of intertwining filamentous forms. Bar, 1 μm.*

FIG. 2. *M. pneumoniae grown on glass cover slips for 48 h. The cover slips with the organisms were first washed with buffer, then fixed. (a) General view showing the filamentous organisms attached to the glass and microcolonies. Bar, 10 μm. (b) Higher magnification showing the individual organisms. The bulbous swellings are very prominent. Bar, 1 μm.*

FIG. 3. *M. pneumoniae grown on glass and treated with 25 μg of trypsin per ml for 10 min. Many swollen cells can be seen (compare with Fig. 1b). Bar, 1 μm.*

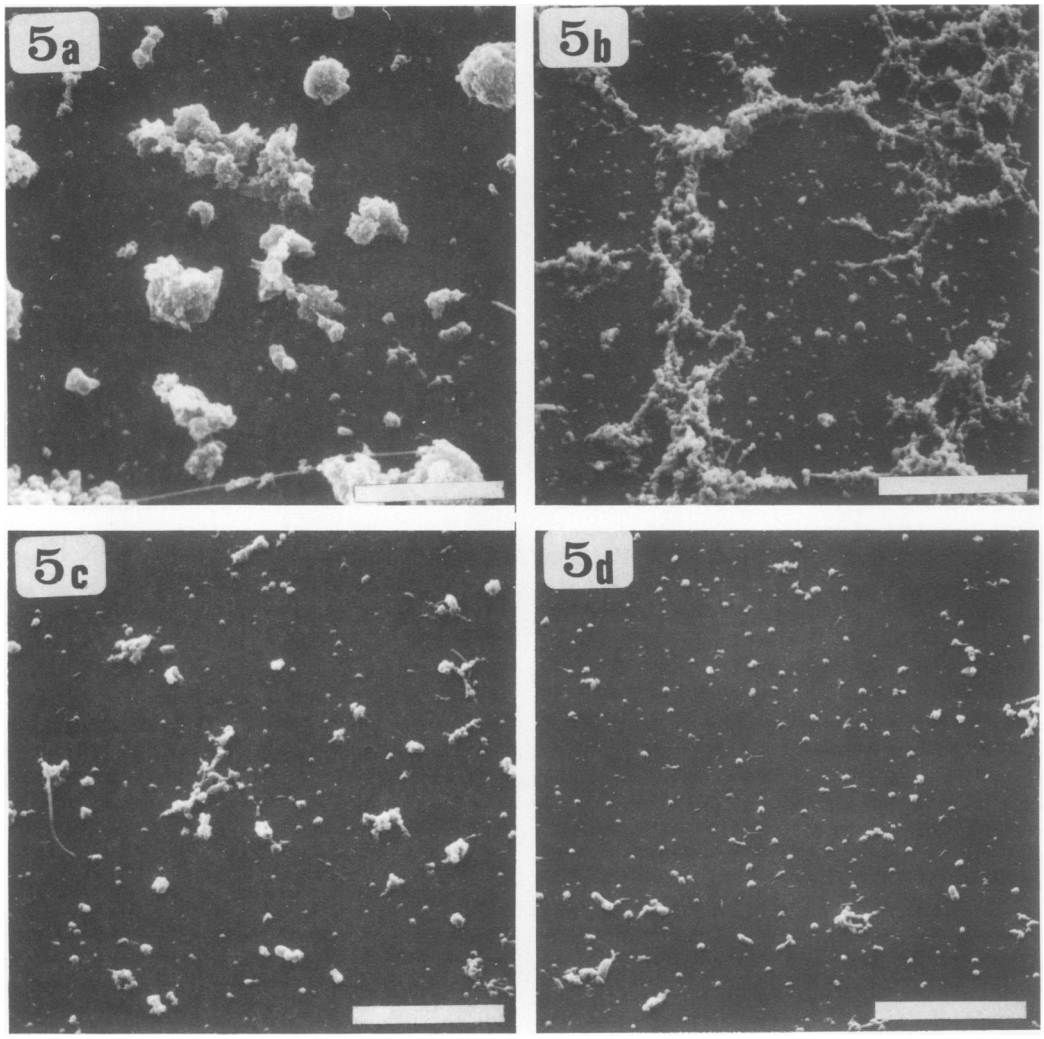

FIG. 5. *Effects of sonication or filtration on M. pneumoniae cell suspensions. (a) Cell suspensions prepared by scraping off the sheet of M. pneumoniae growth on Roux bottles. Most organisms are part of microcolonies. (b) Same suspension after gentle sonication. The microcolonies are in the process of disaggregation. (c) Same suspension as in (a) after filtration through an 8-μm membrane filter. Many single cells and small aggregates, as well as a few filaments can be seen. (d) Same suspension as in (a) after filtration through a fiberglass filter (GF/c). Mostly single coccoid forms. Bar, 10 μm.*

organisms spread on the inert surface, appear to consist of intertwining filaments. Although Biberfeld and Biberfeld (4) stated that the colonies are comprised primarily of coccoid or pear-shaped cells, their electron micrographs of thin sections through colonies suggest the filamentous nature of the cells, in line with the filamentous shape of the organisms revealed in negatively stained preparations of *M. pneumoniae* microcolonies (8).

The peculiar ropelike twisted filaments observed in a few of our *M. pneumoniae* preparations (Fig. 4) have not been described in this

TABLE 1. *Attachment of mycoplasma suspensions to RBC*

Organism and treatment of suspension	Mycoplasmas attached to RBC (% attached of total in suspension)
M. pneumoniae	
Untreated suspension	21.5
Filtered through GF/c filter	5.1
Filtered through 8-μm membrane filter	3.7
Sonicated (40 s)	1.9
M. galisepticum	
Untreated suspension	7.8

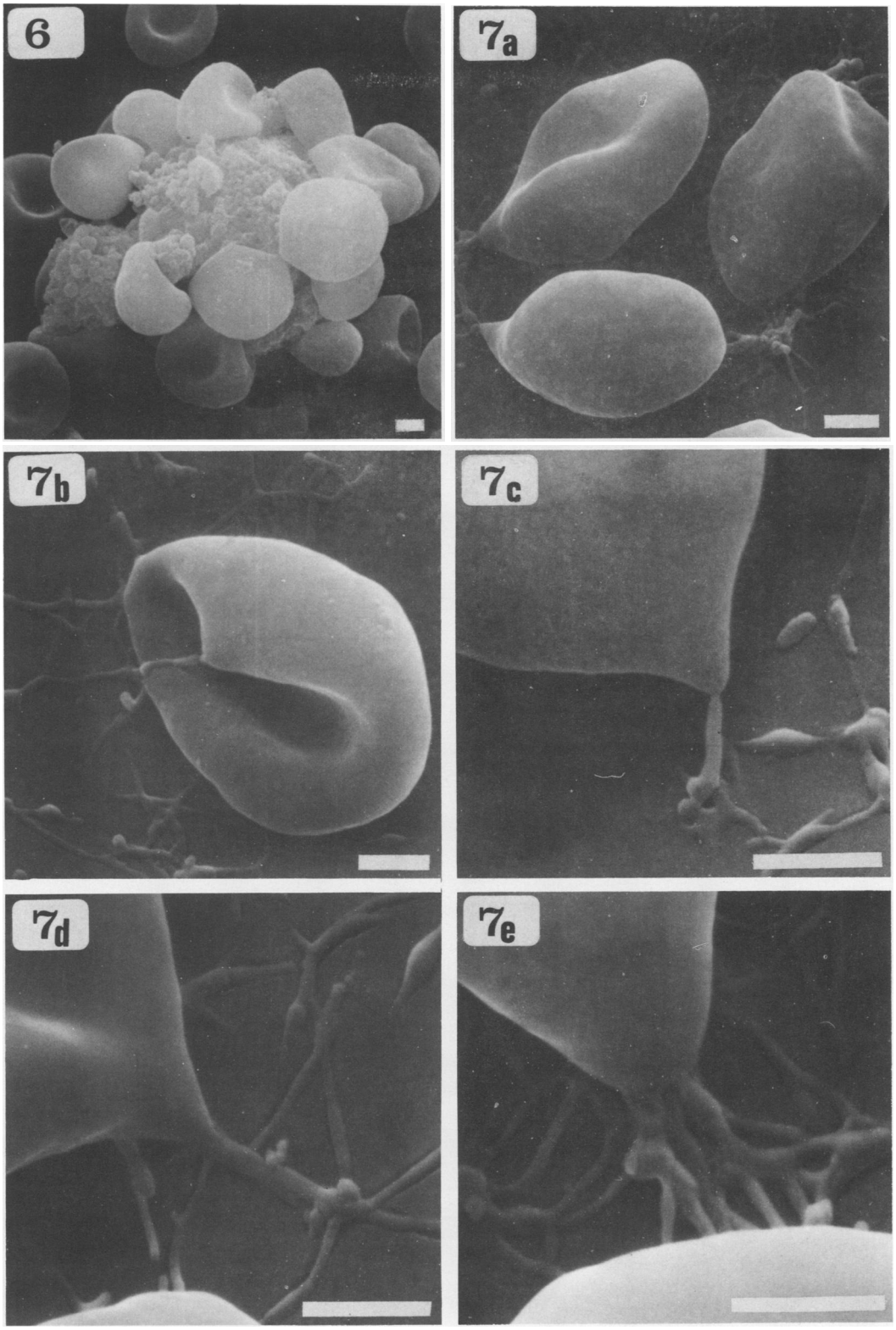

FIG. 6. *RBC adhering to microcolonies of M. pneumoniae in suspension. Some distortion of shape of RBC adhering to the colony can be seen. Bar, 1 μm.*

FIG. 7. *RBC adhering to M. pneumoniae grown on plastic cover slips of Leighton tubes. (a) RBC attaching to filaments of M. pneumoniae. The distorted shape of the RBC, apparently caused by their tight attachment to the mycoplasmas, can be seen. (b) Distortion of the shape of an RBC caused by its attachment to a mycoplasma filament. (c) Attachment of an RBC to the tip of a M. pneumoniae filament. (d and e) Attachment of RBC to M. pneumoniae filaments. The tight nature of the attachment is discernible. Bar, 1 μm.*

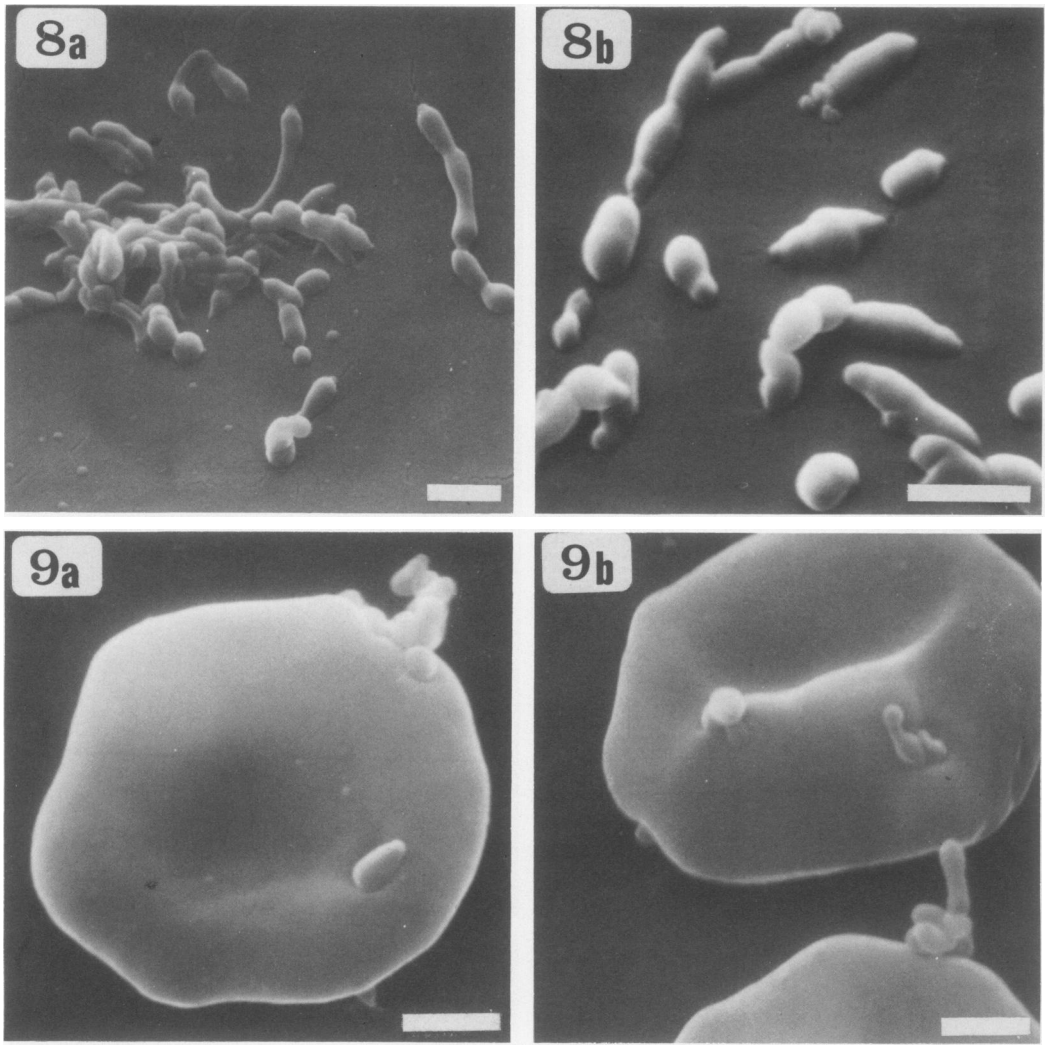

FIG. 8. *M. gallisepticum growing on the plastic cover slips of Leighton tubes. (a) Microcolony. Coccoid or elongated filamentous bodies can be seen. (b) Higher magnification showing the bleblike structures at the tip of the fusiform or teardrop-shaped cells. Bar, 1 μm.*

FIG. 9. *(a and b) M. gallisepticum adhering to RBC in suspension. The tight association of the mycoplasmas with the RBC is evident from the indentations of the RBC surface at the sites of contact with the mycoplasmas. Bar, 1 μm.*

organism thus far, but they were observed in cultures of a ureaplasma (T-strain mycoplasma) by Klainer and Pollack (20). The nature of these forms is yet to be clarified.

The morphology of *M. gallisepticum* observed by us resembles that reported in the SEM study of Gallagher and Rhoades (13). The individual cells show the unique fusiform or teardrop shape and have blebs at the poles. However, our electron micrographs demonstrate that *M. gallisepticum* also resembles other mycoplasmas in its ability to attain a filamentous shape (Fig. 8a). Moreover, branched chains of coccoid bodies (Fig. 8b) can also be observed, so that the postdivisional morphology of *M. gallisepticum* is not basically different from that of other mycoplasmas, supporting our previous proposal based on phase-contrast microscopy (24).

Our SEM study demonstrates the difficulty hampering the quantification of *M. pneumoniae* adherence to RBC in suspensions prepared by scraping off the growth of the organism from glass or plastic surfaces. Most of the organisms in these suspensions are part of microcolonies

(Fig. 5a) which hemadsorb the RBC most effectively (Fig. 6). However, in this case most of the mycoplasmas in the resulting aggregates do not actually come into contact with the RBC, so that the radioactivity values used to measure attachment must be higher than the actual values. Sonication disaggregates the microcolonies, but the fragile wall-less mycoplasmas are damaged even by the briefest sonication period used in the experiments. The very low attachment values attained by the sonicated suspensions (Table 1) apparently reflect cell damage, since mycoplasma adherence is markedly influenced by the metabolic state and structural integrity of the microorganisms (12, 16). Crude lipase was found by Boatman and Kenny (5) to disaggregate *M. pneumoniae* colonies. We did not test this reagent, since Hu et al. (17) showed that it affects *M. pneumoniae* attachment to hamster tracheal explants, probably due to contamination of the crude enzyme preparation with proteolytic enzymes. Trypsin treatment of *M. pneumoniae* abolished its ability to attach to tracheal explants (17) and human RBC (Banai et al., submitted for publication). Our efforts to remove the colonies by filtration of the suspensions proved successful; the attachment values obtained with the filtered suspensions probably represent the true values. These are close to the attachment values of untreated *M. gallisepticum* suspensions, which are essentially devoid of microcolonies (Table 1).

The attachment of *M. gallisepticum* to RBC visualized by SEM (Fig. 9) appears to be tight, as suggested by the indentations of the RBC surface at the contact sites. Similar indentations were also observed by Apostolov and Windsor (2) and by us (unpublished results) in ultrathin sections of *M. gallisepticum* attached to RBC. The tight nature of the attachment of *M. gallisepticum* to RBC was also demonstrated by our failure to detach the organisms by prolonged washing (3). Nevertheless, the micrographs do not allow one to conclude whether or not the organisms attach to the RBC through their peculiar bleb structure, as suggested by Zucker-Franklin et al. (29) and by Uppal and Chu (27). Transmission electron microscopy of thin sections is clearly required to resolve this issue.

The interaction between individual *M. pneumoniae* filaments and RBC was best studied with RBC attaching to the surface growth of *M. pneumoniae* on the plastic cover slips of Leighton tubes. Our micrographs demonstrate attachment of a single *M. pneumoniae* filament through its tip to the RBC—an attachment sufficiently tight to cause considerable shape distortion of the RBC. These electron micrographs may be taken in support of the role of the tip structure in adherence of *M. pneumoniae* to RBC, at variance with the conclusion of Brunner et al. (7). However, as in the case of *M. gallisepticum*, definite conclusions must await additional studies by transmission electron microscopy of thin sections. The tips of *M. pneumoniae* filaments look as if they fuse with the RBC membrane. Whether this reflects true fusion of the parasite and host membranes cannot be decided on the basis of the SEM micrographs alone. Since there is no barrier (i.e., a cell wall) separating the plasma membrane of the mycoplasma from that of its host, the plausibility of fusion of the two membranes has attracted the attention of many investigators. Obviously, if fusion does occur, a wide variety of potentially cytotoxic proteins and lipids can be introduced directly into the host cells. Some evidence for the fusion of *M. gallisepticum* with erythrocytes has been provided by Apostolov and Windsor (2) with transmission electron microscopy. The more recent observation of *M. hyorhinis* capping on the surface of mouse lymphocytes (26) and exchange of membrane antigens between murine T-lymphoblastoid cells and *M. hyorhinis* (28) reflect the intimate association between the membrane of the parasite and its host. Our SEM observations encourage further studies on the possible fusion between eucaryotic and mycoplasma cell membranes.

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