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Interaction of Virulent *Mycoplasma pneumoniae* with Hamster Tracheal Organ Cultures

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Exposure of hamster tracheal organ cultures to virulent *Mycoplasma pneumoniae* leads to alterations in ribonucleic acid (RNA) and protein biosynthesis and metabolism of the respiratory epithelium. An examination of the turnover rates of RNA and protein in infected tracheal organ cultures indicates that the rates of degradation of both prelabeled host cell RNA and protein are similar to those of uninfected controls. Infected tracheal organ cultures shifted to a nonpermissive medium within 24 h after infection and further incubated in the nonpermissive medium for 72 or 96 h behaved as normal uninfected cultures in terms of metabolic precursor uptake. Under these conditions, mycoplasmas remained attached to the respiratory epithelium. Cell membranes prepared from virulent mycoplasmas by several procedures neither attached to nor altered the metabolic activity of tracheal cultures. These data indicate that the intimate contact between virulent mycoplasmas and the respiratory epithelium does not alone account for the subsequent interruption of host cell metabolism but must be accompanied by continued multiplication and biochemical function of attached mycoplasmas.

The specificity and critical nature of attachment of microbial pathogens to mucosal surfaces have been demonstrated in numerous bacterial and viral diseases (1, 5, 8, 11, 12, 19). During *Mycoplasma pneumoniae* infection, the interaction of virulent mycoplasmas with the respiratory epithelium and the subsequent development of ciliostasis and other cytopathology occur in human and animal tissues (5, 6). It appears that a highly differentiated tiplike structure on *M. pneumoniae* organisms permits the orientation and localization of virulent mycoplasmas to specific neuraminidase-sensitive receptors on the respiratory epithelium (5, 16). In a recent study using hamster tracheal organ cultures (16), we have further defined the kinetics of attachment and other properties of the host cell-mycoplasma interaction, reinforcing the idea that there are unique and specialized requirements for the initiation of *M. pneumoniae* disease. Once attachment has occurred, virulent mycoplasmas multiply and parasitize the respiratory tissue, leading first to alterations in host cell macromolecular synthesis and carbohydrate metabolism and then to tissue cytopathology (13). In the latter study, we observed decreased rates of host cell ribonucleic acid (RNA) and protein synthesis early in the experimental infection and concluded that biochemical lesions in transcription or translation preceded tissue necrosis. However, at that time we were uncertain as to the role played by host cell or mycoplasma hydrolytic enzymes in effecting the decreased accumulation of radiolabeled macromolecules by respiratory tissues. Thus it was important to determine the extent of hydrolytic degradation of host cell macromolecules during infection.

In addition, Gabridge et al. (10) have reported that cell membranes prepared from virulent strains of *M. pneumoniae* are cytotoxic for hamster tracheal organ cultures. These authors concluded that a toxic factor associated with the mycoplasma cell membrane is responsible for *M. pneumoniae* pathogenicity. However, we have reported that only metabolically active mycoplasmas are capable of attaching via the specialized tip structure to receptor sites on the epithelium of hamster trachea (16). We have also shown that alterations in host cell metabolism after *M. pneumoniae* attachment require continued viability and/or metabolic activity of *M. pneumoniae* organisms (13). In this paper, we further characterize the host cell-mycoplasma interaction, reemphasizing the role of intact, viable, and biosynthetically active virulent mycoplasmas in the development of disease.

MATERIALS AND METHODS

Radioisotopes and reagents. [^{3}H]orotic acid (specific activity, 11.5 Ci/mmol) was purchased from International Chemical and Nuclear Corp., Irvine,

Calif.; [^{3}H]uridine (specific activity, 19.0 Ci/mmol) from Nuclear Dynamics Inc., El Monte, Calif.; ^{14}C-labeled amino acid mixture (specific activities of 100 to 500 Ci/mmol) from Schwarz/Mann, Orangeburg, N.Y.) and carrier-free Na^{125}I (specific activity, 17 Ci/mg) from New England Nuclear Corp., Boston, Mass. Glucose oxidase (A grade) and lactoperoxidase (purified grade) were obtained from Calbiochem Co., La Jolla, Calif. All other reagents were of chemically pure grade.

Growth of organisms. Monolayer cultures of virulent *M. pneumoniae* (M129-B10) were grown on glass in Hayflick medium as previously described (16). After incubation at 37 C in 5% CO_2 with air for 48 h, organisms were gently scraped into 2.5 to 5.0 ml of either fresh Hayflick medium supplemented with 20% agamma horse serum and 10% yeast dialysate or Eagle minimum essential medium (MEM) supplemented with 20% fetal calf serum and 10% yeast dialysate. Clumps of mycoplasmas were dispersed by passing the suspension three times through a 25-gauge needle.

Analysis of macromolecular degradation during infection. Tracheal rings were prepared as previously described (13) and incubated in 3.0 ml of MEM containing either [^{3}H]uridine (100 μCi) or a mixture of ^{14}C-labeled amino acids (5 μCi) for 24 h. Rings were rinsed and incubated in 3.0 ml of fresh MEM without isotope for an additional 24 h to reduce the soluble radioactive pools before infection. Infection was initiated by submerging tracheal rings in a drop of log-phase *M. pneumoniae* culture for 4 h. Control cultures were immersed in fresh Hayflick medium. Then rings were washed twice in MEM and incubated in fresh Hayflick medium with daily changes of medium. At specific time intervals, individual rings were removed, rinsed twice in phosphate-buffered saline (PBS), and placed in small glass tubes containing 0.1 ml of 1% sodium dodecyl sulfate to solubilize the cells. Tubes were mixed briefly and kept at 37 C for 10 min. A 25-μl volume of 0.5% bovine serum albumin was added to individual tubes followed by 0.1 ml of 15% cold trichloroacetic acid. Tubes were mixed again and placed in an ice bath for 30 min. The trichloroacetic acid-insoluble fractions were collected on 0.22-μm membranes (Millipore Corp.), washed with 5 ml of 5% cold trichloroacetic acid, dried, and counted. Radioactive values presented in all tables are the average of six tracheal rings from at least two separate experiments.

Measurement of macromolecular biosynthesis after infection. Control and infected tracheal rings were removed from Hayflick medium at specific intervals and rinsed in serum-free MEM. Then individual rings were placed in microtiter plate wells containing 50 μl of MEM with either 1 μCi of [^{3}H]orotic acid alone or 0.3 μCi of a ^{14}C-labeled amino acid mixture supplemented with 80 μg of erythromycin per ml. This concentration of antibiotic selectively inhibited protein synthesis by mycoplasmas. Incubation was continued for 4 h before trichloroacetic acid-insoluble fractions were counted by liquid scintillation spectrometry as previously described by us (2, 13).

Preparation of *M. pneumoniae* membrane. Cell membrane of mycoplasmas was prepared by essentially the procedures of Gabridge et al. (10) as adopted from Razin and Rottem (18). Glass-grown virulent mycoplasmas were washed five times with PBS (pH 7.2) to remove serum and then were scraped into 5.0 ml of deionized water. The *M. pneumoniae* cell suspension was transferred to a sterile vial containing 1 g of fine glass beads (type 1, 75 to 100 μm; Sigma Chemical Co., St. Louis, Mo.) and sonicated for 5 s at 15 kH_Z (Branson Sonifier, model LS75, Great Neck, N.Y.) before freeze-thawing. These steps greatly enhanced the rate of cell breakage, thereby increasing the amount of recovered mycoplasma membrane. The preparation was then centrifuged at 500 $\times$ *g* for 10 min, and the supernatant containing the cell membrane fraction was carefully recovered. Membranes were collected by centrifugation at 100,000 $\times$ *g* for 30 min, washed six times in alternate solutions of 0.5 M NaCl and β-buffer (10), resuspended in PBS, and stored at -20 C. Membranes were also prepared by treatment with digitonin and osmotic shock by the methods of Razin and Rottem (18). Aseptic techniques were used throughout the preparation, and samples were routinely examined for the presence of mycoplasma and other bacterial contaminants. In no case were viable microorganisms isolated.

Iodination of mycoplasmas. Iodination of *M. pneumoniae* by the procedure of Hynes (14) was performed in monolayer cultures. Cells were washed five times with PBS, and 5 to 10 ml of PBS supplemented with 2 mM glucose was then added. Carrier-free Na^{125}I was introduced to a final concentration of 50 to 100 μCi/ml. The reaction was initiated by addition of lactoperoxidase and glucose oxidase at concentrations of 20 μg/ml and 0.1 IU/ml, respectively. The reaction was allowed to continue for 10 min at room temperature with occasional swirling. The medium was quickly removed after incubation, and ^{125}I labeling was stopped by the addition of phosphate-buffered iodine (similar to PBS with NaCl replaced by NaI). Iodinated organisms were washed two more times with phosphate-buffered iodine, followed by three washes with PBS. The washed cells were scraped off the glass and suspended in deionized water. Cell membranes from iodinated mycoplasmas were prepared in the same manner as previously described, except that 2 mM phenylmethylsulfonyl fluoride was added in phosphate-buffered iodine to inhibit proteases.

Radioautography and electron microscopy. Tracheal rings were fixed in 2.5% glutaraldehyde-2.0% *p*-formaldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature. The rings were washed and secondary fixation was carried out in 1% OsO_4-Veronal acetate buffer, pH 7.3, for 1.5 h at 4 C, followed by dehydration and embedding in Epon (16). For radioautography, 0.25-μm sections were cut on an LKB ultramicrotome with a glass knife and mounted on microscope slides. The slides were coated with Kodak NTB-2 nuclear tract emulsion, stored in the dark for 1 to 3 weeks at 4 C, developed in Kodak D-19, and then stained with Richardson stain. For electron microscopy, thin sections were

cut with a diamond knife, stained with uranyl acetate, and mounted on grids for inspection in an AEI electron microscope.

RESULTS

Degradation of prelabeled macromolecules after mycoplasma infection. As shown in Table 1, the rate of turnover of prelabeled RNA in infected tracheal rings after 72 h of infection was similar to that seen in uninfected controls. Also, radioactive determinations performed on samples of incubation medium from both control and infected rings during the 72-h period indicated a similar release of radioisotope from tissue into the surrounding medium (data not shown). Other data (13) demonstrated that during infection of tracheal organ cultures, *M. pneumoniae* did not utilize preformed or degraded host cell RNA for the biosynthesis of mycoplasmal RNA. The slight decrease in radioactivity observed in infected rings probably results from the loss of respiratory cells during infection (see Fig. 1B). In contrast, the rate of RNA synthesis in mycoplasma-infected tracheal rings was reduced to 20% of that seen in the controls by 72 h. A similar relationship was observed when the rates of degradation and synthesis of protein were compared (Table 2). These observations indicate that decreased rates of macromolecular synthesis by infected tracheal organ cultures do not result from an accelerated degradation of preexisting host cell RNA or protein.

Effect of mycoplasma cell membrane on the uptake of precursors. Since Gabridge et al. (10) had reported cytotoxicity of *M. pneumoniae* membranes for hamster tracheal organ cultures, we tested to see whether mycoplasma cell membrane prepared by several procedures (see Materials and Methods) inhibited the metabolism of the respiratory epithelium. At specific time intervals, the rate of uptake of

TABLE 1. *Rates of turnover and synthesis of RNA in control and M. pneumoniae-infected tracheal organ cultures*

Incubation (h)	Counts/min per ring					
	Turnover[a]			Synthesis[b]		
	Control	Infected	% Infected/control	Control	Infected	% Infected/control
24	136,855	121,179	88.5	520	348	66.9
48	111,971	106,829	95.5	334	122	36.5
72	77,145	67,626	87.6	441	90	20.4

[a] Tracheal rings were prelabeled with 100 μCi of [^{3}H]uridine in 3.0 ml of MEM for 24 h, and then rinsed and incubated in fresh MEM without isotope for 24 additional h before infection.

[b] Tracheal rings were incubated in MEM at 37 C overnight before infection. At appropriate times, control and infected rings were exposed to [^{3}H]orotic acid (1 μCi) for 4 h, and radioactivity was determined as described in Materials and Methods.

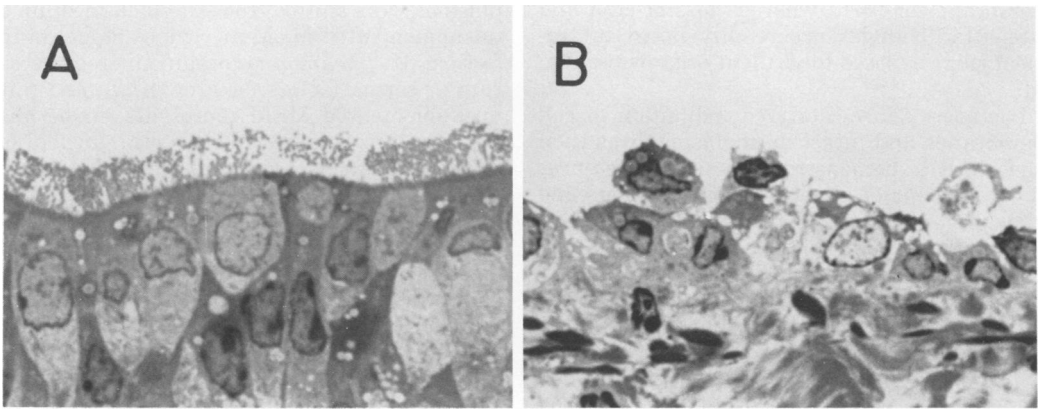

FIG. 1. *Hamster tracheal organ cultures incubated with (A) membranes (40 μg of protein/ml) prepared from virulent M. pneumoniae for 96 h in MEM supplemented with 20% fetal calf serum and 10% yeast dialysate; (B) viable virulent M. pneumoniae organisms for 96 h in the same medium. Note loss of cilia and extensive cell damage in (B). (×2,500)*

TABLE 2. *Rates of turnover and synthesis of protein in control and M. pneumoniae-infected tracheal organ cultures*

Incubation (h)	Counts/min per ring					
	Turnover[a]			Synthesis[b]		
	Control	Infected	% Infected/control	Control	Infected	% Infected/control
24	24,272	22,276	91.8	3,870	2,054	53.1
48	16,905	14,672	86.8	3,516	1,539	43.8
72	11,996	9,545	79.6	4,262	742	17.5

[a] Tracheal rings were prelabeled with 5 μCi of ^{14}C-labeled amino acid mixture in 3.0 ml of MEM for 24 h, and then rinsed and incubated in fresh MEM without isotope for 24 additional h before infection.

[b] Tracheal rings were incubated in MEM at 37 C overnight before infection. Both control and infected rings were incubated for 1 h with erythromycin (80 μg/ml) followed by a 4-h incubation with 0.2 μCi of ^{14}C-labeled amino acid mixture before acid-insoluble radioactivity was determined.

[^{3}H]uridine was monitored as an indicator of alterations in metabolic function. The results are summarized in Table 3. It is apparent that no difference exists in the rate of uptake of [^{3}H]uridine between the tracheal rings incubated with mycoplasma cell membrane and controls during 120 h of incubation.

Attachment of *M. pneumoniae* and mycoplasma cell membrane to tracheal epithelium. When tracheal rings were incubated in serum-supplemented MEM containing cell membrane (20 to 50 μg of protein/ml) prepared from virulent mycoplasmas as described in Materials and Methods, ciliary activity appeared similar to control cultures incubated in serum-supplemented MEM medium alone. However, rings exposed to live mycoplasmas showed a significant reduction in ciliary activity within 48 h, with ciliostasis occurring by 96 h after the initial infection. As seen in Fig. 1A, cilia are intact and epithelial cells appear normal in tracheal rings incubated with mycoplasma cell membrane for 96 h, whereas loss of cilia and cytopathic changes are readily noted in tracheal rings exposed to virulent organisms (Fig. 1B).

Lactoperoxidase-catalyzed iodination of cell membranes and intact mycoplasmas was then performed to demonstrate possible differences in attachment of membrane preparations and whole organisms. Radioautography of tracheal rings treated with iodinated mycoplasma cell membrane (Fig. 2A) indicates that no attachment of cell membrane to the respiratory epithelium occurs. Figure 2B shows that intact ^{125}I-labeled mycoplasmas readily attach to the respiratory epithelium as demonstrated by the numerous silver grains concentrated over the respiratory tissue. Plate counts of mycoplasmas labeled with ^{125}I indicated no loss in viability.

Influence of culture medium on *M. pneumoniae* infection. To further test the possibility that mediation of host cell injury requires metabolically active and attached mycoplasmas, tracheal rings exposed to virulent mycoplasmas were shifted from Hayflick medium to unsupplemented MEM at various stages of infection. *M. pneumoniae* organisms require serum or serum factors for growth (3), and thus unsupplemented MEM represents nonpermissive growth conditions. The total incubation time for all tracheal rings after initial exposure to virulent mycoplasmas was 96 h. At the end of the incubation, tracheal rings were pulsed for 4 h at 37 C with either [^{3}H]orotic acid or ^{14}C-labeled amino acids plus erythromycin (80 μg/ml) to monitor changes in metabolic activity. The results are shown in Tables 4 and 5. It is important to note that the shifting of infected tracheal rings after 24 h from a permissive (Hayflick medium) to a nonpermissive (MEM) medium prevents the development of altered host cell RNA and protein synthesis. However, a significant decrease in the uptake of metabolic precursors is observed when infected tra-

TABLE 3. *Uptake of [^{3}H]uridine in tracheal rings treated with cell membrane prepared from virulent M. pneumoniae (M129-B10)*[a]

Incubation (h)	Counts/min per ring		% Treated/control
	Control	Membrane-treated	
24	17,794	20,552	115.5
48	25,751	34,986	135.9
72	22,101	17,739	80.3
96	19,246	19,562	101.6
120	18,670	24,419	130.7

[a] Tracheal rings were incubated in MEM alone or MEM containing 20 μg of protein/ml of *M. pneumoniae* membrane. All media were replaced after 48 and 96 h of incubation with appropriate fresh membrane-containing or -deficient MEM. Procedures for labeling were as previously described (13).

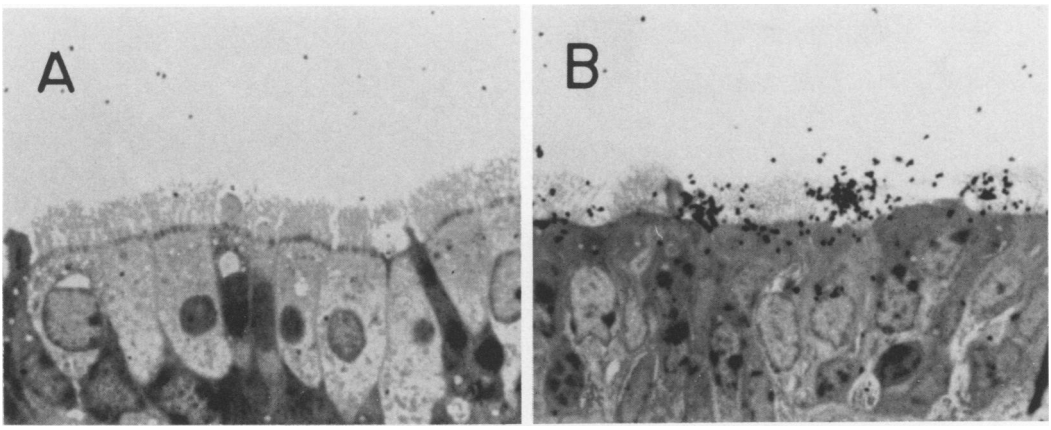

FIG. 2. *Radioautographs of hamster tracheal organ cultures exposed to (A) 20 μg of protein/ml of ^{125}I-labeled membranes (equivalent to 2 × 10^5 counts/min) prepared from virulent M. pneumoniae; (B) ^{125}I-labeled viable virulent M. pneumoniae organisms (equivalent to 7 × 10^4 counts/min). Black emulsion grains distributed along the luminal surface of the epithelial cells represent attached mycoplasmas (B). (×2,500)*

TABLE 4. *RNA synthesis in hamster tracheal rings after shifting to nonpermissive media*[a]

Incubation in Hayflick medium (h)	Counts/min per ring		% Infected/ control
	Control	Infected	
0	6,043	6,415	106
24	6,180	6,270	101
48	5,108	2,370	74
72	5,560	741	13

[a] Control and infected tracheal rings were incubated in Hayflick medium for specific intervals before their transfer to unsupplemented MEM. All tracheal rings were incubated for a total of 96 h. Procedures for labeling with [^{3}H]orotic acid were as described in Materials and Methods.

TABLE 5. *Protein synthesis in tracheal rings after shifting to nonpermissive media*[a]

Incubation in Hayflick medium (h)	Counts/min per ring		% Infected/ control
	Control	Infected	
0	15,180	13,430	88
24	15,048	12,921	86
48	14,816	8,829	60
72	14,170	4,544	32

[a] Procedures are as described in Table 4, except that rings were labeled with 0.2 μCi of ^{14}C-labeled amino acid in the presence of erythromycin for 4 h.

cheal rings that have been incubated in Hayflick medium for 48 h or longer are shifted to MEM. Electron microscopy (Fig. 3A and B) reveals that mycoplasmas still remain attached via the tip structure to respiratory tissue even after incubation in a nonpermissive growth medium for 72 and 96 h. Thus, the initial host cell-mycoplasma interaction appeared stable throughout the experiment. In addition, these mycoplasmas are capable of growth when infected tracheal rings incubated in nonpermissive growth medium for 96 h are transferred back to a permissive growth medium (data not shown). Figure 3C demonstrates the loss of cilia and other cytopathology in infected tracheal rings that have been incubated in permissive medium for the entire 96-h period. Mycoplasmas can be seen attached to the remaining respiratory cells.

DISCUSSION

We previously described alterations in respiratory cell metabolism after infection of hamster tracheal organ cultures with virulent *M. pneumoniae* (13). Significant decreases in both RNA and protein synthesis occurred in epithelial cells within 24 h after infection and preceded reduction in precursor uptake and cytopathology. Therefore, it appeared that initial biochemical lesions in the respiratory epithelium after *M. pneumoniae* infection occurred at the level of macromolecular biosynthesis. In the present study, an examination of the turnover rates of RNA and protein in infected tracheal cultures indicates that the rates of degradation of both prelabeled RNA and protein are similar to those of uninfected trachea controls (Tables 1 and 2). These observations suggest that reduction in the rates of RNA and protein biosynthesis observed in infected host cells does not result from increased activity of hydrolytic enzymes. However, these data do not rule out the possibility that host cell deoxyribonucleic acid may be highly sensitive to degradation after the mycoplasma-host cell interaction.

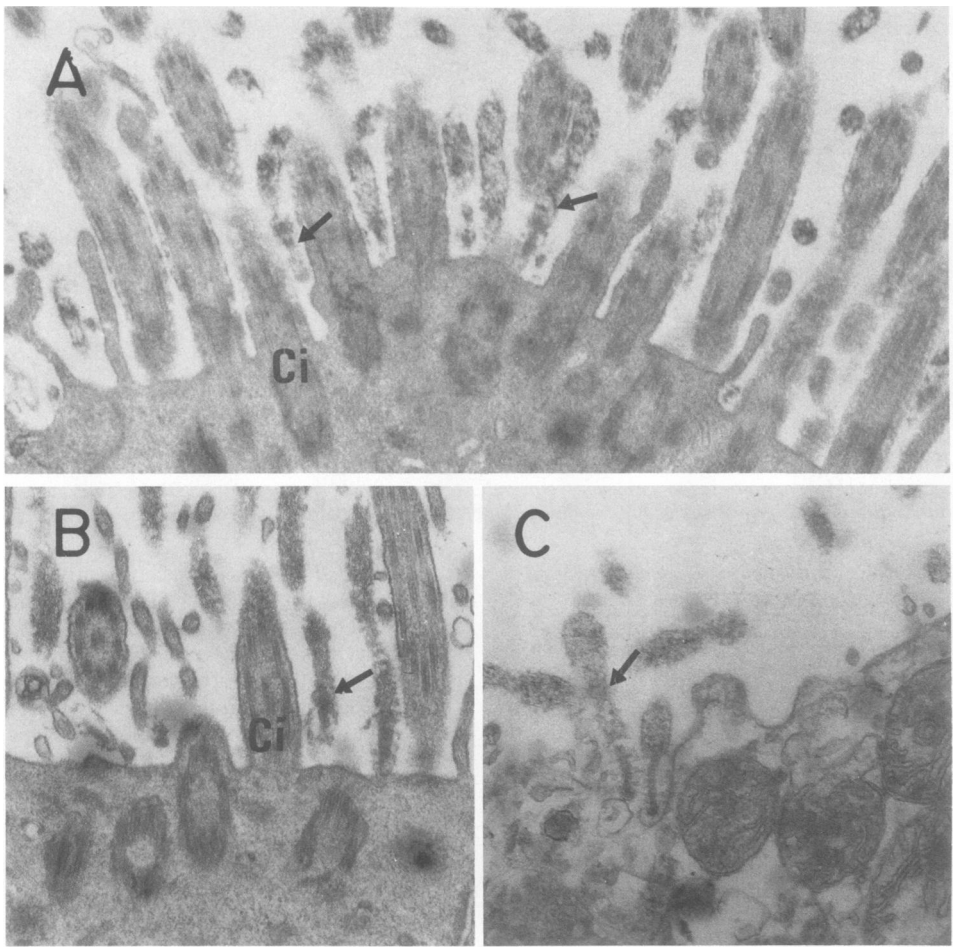

FIG. 3. *Electron micrographs of infected hamster tracheal organ cultures. (A) Tracheal ring exposed to virulent M. pneumoniae for 4 h in Hayflick medium and then incubated in nonpermissive MEM for 96 h. (B) Tracheal ring treated as in (A), incubated in Hayflick medium for 24 h, and then shifted to nonpermissive medium for 72 h. Note the maintenance of the respiratory epithelium and cilia in (A) and (B) and contrast with (C). (C) Tracheal ring treated as in (A) and then incubated in Hayflick medium for 96 h. Note loss of cilia and cellular destruction in (C). Arrows indicate mycoplasmas attached to the respiratory epithelium. Ci, Cilia. (×35,000)*

In previous studies (4, 5), we demonstrated that infection of tracheal organ culture with *M. pneumoniae* begins with attachment of virulent mycoplasmas via a tip structure to receptors on respiratory cells, followed several days later by ciliostasis and progressive destruction of the tracheal epithelium. Avirulent *M. pneumoniae* organisms derived from the same parent strain do not attach to epithelial cells and produce no cytopathic effects (5). Furthermore, colonies of avirulent mycoplasmas grown on solid medium also lose their ability to absorb erythrocytes, a major in vitro characteristic of virulent *M. pneumoniae* (15). Although these observations strongly suggest that the ability of *M. pneumoniae* to attach to epithelial cells of the respiratory tract is a prerequisite for disease production, the present data indicate that intimate contact between virulent mycoplasmas and the respiratory epithelium alone does not account for the subsequent interruption of host cell macromolecular synthesis and tissue cytopathology. Tracheal organ cultures shifted to a nonpermissive medium within 24 h after infection and further incubated in a nonpermissive medium for 72 or 96 h behaved as normal uninfected cultures (Tables 4 and 5). Under these conditions, mycoplasmas still remained attached to the respiratory epithelium (Fig. 3A and B) and were capable of growth if trans-

ferred to a permissive growth medium. These observations support the concept that *M. pneumoniae* infection is a two-step process. The initial and specific attachment of mycoplasmas via a tip structure to receptors on respiratory cells allows virulent mycoplasmas to colonize the host tissue. The metabolic alterations and other cytopathology that follow the initial parasite-host cell interaction appear to result from the multiplication of mycoplasmas accompanied by possible membrane perturbation, nutritional parasitism, and/or introduction of certain toxic factors into sensitive cells. The nature of such "toxic factors" is unknown but presumably would be dose-dependent and be generated only by metabolically active and attached mycoplasmas. The latter point is substantiated since decreased uptake of metabolic precursors by host cells can be prevented by addition of erythromycin (13) or shifting infected tracheal rings to a nonpermissive medium at early and intermediate stages of infection (Tables 4 and 5).

Gabridge et al. have reported (10) that membrane preparations of *M. pneumoniae* induce gross cytopathology in tracheal organ cultures similar to that observed after infection with viable organisms. We have been unable to confirm these findings using similar membrane preparations, mycoplasma strains, and experimental techniques. We observed no significant decrease in ciliary activity or loss of epithelium in tracheal organ cultures incubated with *M. pneumoniae* membrane for 96 to 120 h when compared with untreated controls (Fig. 1A and B). Neither could we detect any significant reduction in the rate of metabolic precursor uptake in such membrane-treated organ cultures (Table 3). Furthermore, cell membranes prepared by these techniques tend to form aggregates or clumps when resuspended in PBS, as evidenced by light microscopy, and do not attach to the respiratory epithelium (Fig. 2A). Even if *M. pneumoniae* membranes were cytotoxic, such large aggregates conceivably would be unable to reach the luminal surface at the base of the cilia of the respiratory epithelium, which has been shown to be the attachment site for virulent *M. pneumoniae* in both tracheal organ cultures (4, 5, 6) and human sputum obtained during the acute stages of mycoplasma pneumonia (7). In addition, we recently demonstrated that only metabolically active *M. pneumoniae* are able to attach to the respiratory ciliated epithelium (16), suggesting that the mobility of the organisms and other mycoplasma functions are required for attachment. Therefore, at this time, experimental differences relating to membrane cytotoxicity as reported by Gabridge et al. (10) cannot be reconciled. A recent report by Daniel and Wolf (9) indicates that HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at a concentration of 25 mM causes reduced ciliary movement and altered surface appearance of the epithelium of hamster tracheal organ cultures within a few days. Similar concentrations of HEPES were used by Gabridge et al. in their studies on the cytotoxicity of *M. pneumoniae* membranes (10). Other possibilities might also be considered. For example, since *Mycoplasma* species are unable to synthesize long-chain fatty acids, variations in medium content could significantly alter the lipid composition of *M. pneumoniae* membrane (17). Also growth and age of the cultures and enzyme activities of isolated membranes are likely to vary between laboratory preparations. Nonetheless, our data lend support to the critical nature and function of the intact, tiplike appendage, as well as the metabolic integrity of virulent *M. pneumoniae* organisms after their specific attachment to membrane receptors on respiratory tissue.

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