Mycoplasma pulmonis Inhibits Electrogenic Ion Transport across Murine Tracheal Epithelial Cell Monolayers

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Murine chronic respiratory disease is characterized by persistent colonization of tracheal and bronchial epithelial cell surfaces by Mycoplasma pulmonis, submucosal and intraluminal immune and inflammatory cells, and altered airway activity. To determine the direct effect of M. pulmonis upon transepithelial ion transport in the absence of immune and inflammatory cell responses, primary mouse tracheal epithelial cell monolayers (MTEs) were apically infected and assayed in Ussing chambers. M. pulmonis-infected MTEs, but not those infected with a nonmurine mycoplasma, demonstrated reductions in amiloride-sensitive Na+ absorption, cyclic AMP, and cholinergic-stimulated Cl− secretion and transepithelial resistance. These effects were shown to require interaction of viable organisms with the apical surface of the monolayer and to be dependent upon organism number and duration of infection. Altered transport due to M. pulmonis was not merely a result of epithelial cell death as evidenced by the following: (i) active transport of Na+ and Cl−, albeit at reduced rates; (ii) normal cell morphology, including intact tight junctions, as demonstrated by electron microscopy; (iii) maintenance of a mean transepithelial resistance of 440 Ω/cm²; and (iv) lack of leakage of fluid from the basolateral to the apical surface of the monolayer. Alteration in epithelial ion transport in vitro is consistent with impaired pulmonary clearance and altered airway function in M. pulmonis-infected animals. Furthermore, the ability of M. pulmonis to alter transport without killing the host cell may explain its successful parasitism and long-term persistence in the host. Further study of the MTE-M. pulmonis model should elucidate the molecular mechanisms which mediate this reduction in transepithelial ion transport.

Mycoplasmas, the smallest free-living prokaryotes, continue to be a significant cause of respiratory infections in a variety of animals, including humans (44). Their limited biosynthetic capability dictates that these organisms must both colonize and parasitize epithelial cell surfaces. It is therefore surprising that mycoplasmas produce diseases that are slowly progressing and chronic and yet often clinically inconspicuous. The molecular mechanisms responsible for this tenuous truce between the pathogen and the host have not yet been identified. Murine respiratory mycoplasmosis, a naturally occurring respiratory disease in laboratory rats and mice caused by Mycoplasma pulmonis (6, 8) and characterized by chronic, often lifelong tracheitis, bronchopneumonia, and bronchiectasis (7, 23, 28), would seem to be an ideal model with which to study these processes, Na+ absorption, and accumulation of mucus (23) suggest that M. pulmonis may compromise the ability of the epithelial cells to absorb and secrete fluid and electrolytes.

Airway epithelial cells possess two major active transport processes, Na+ absorption and Cl− section. Water, in turn, osmotically follows the transepithelial movement of these ions, thereby providing a fluid film between the mucus layer and the epithelial cell surface. The depth and composition of this fluid microenvironment must be carefully regulated to allow the exchange of gases and the humidification of the airway epithelium and to ensure proper mucociliary clearance (47, 49). The consequences of impaired fluid and electrolyte transport in the airways are strikingly apparent in cases of cystic fibrosis, a recessive genetic disease resulting from the loss of epithelial chloride channels (31, 48). The studies reported here were designed to determine whether M. pulmonis infection alters electrolyte transport across the murine tracheal epithelium.

The effect of bacteria upon mammalian epithelia in the absence of immune and inflammatory cells can be systemati cally evaluated by using the Ussing chamber model. Short-circuit current (Isc) studies have helped determine the mechanism by which cholera toxin (18) and other enterotoxins affect the intestinal mucosa (17, 35). Study of various types of epithelial cells by using the Ussing chamber model has also resulted in the identification of numerous microbial substances capable of directly altering the ion transport capacity of the airway epithelium (3, 20, 41).

In the present study, control and M. pulmonis-infected mouse tracheal epithelial cells (MTEs) were evaluated in Ussing chambers. The results clearly show that M. pulmonis infection directly alters epithelial ion transport and that this alteration is species specific, dose and time related, and dependent upon the association of viable organisms with the apical cell surface.

MATERIALS AND METHODS

Mycoplasmas. Experiments were performed with either a virulent strain of M. pulmonis, CT, originally isolated from a mouse with respiratory mycoplasmatisis (14), or with Mycoplasma fermentans incognitus, a mycoplasma of human origin provided by Shyh-Ching Lo at the Armed Forces Institute of Pathology, Washington, D.C. Organisms were grown, harvested, and stored as previously described (46). M. pulmonis was cultured in mycoplasma broth base (Difco) supplemented with heat-inactivated and filtered horse serum (HyClone Labora-
tories, Logan, Utah) and glucose. After static incubation at 37°C, cultures were divided into 1-ml aliquots and stored at −70°C. Quantitation of mycoplasmas was performed as previously described (1). Specimens were diluted in Hayflick’s broth by serial 10-fold steps (color-changing units [CCU]), and 0.01 ml of each dilution was spread onto two different monoculture antibiotic plates. The dilutions and inoculated plates were incubated at 37°C for 6 to 8 days, and the number of CFU was determined. For quantification of organisms associated with MTEs and demonstration of their growth over time, filters apically inoculated with M. pulmonis for 6 h were washed twice to remove any adhered organisms and then incubated at 37°C. At 24, 48, 72, and 96 h postinoculation, mycoplasma-infecting filters were aseptically removed and vortexed in mycoplasma media, and the number of CFU was determined.

Antigen retrieval procedure. Six-week-old CD-1 mice maintained in Trexler-type plastic film isolators were used in these studies. The pathogen-free status of the animals was determined and then incubated at 37°C. At 24, 48, 72, and 96 h postinoculation, mycoplasma-infected filters were aseptically removed and vortexed in mycoplasma media, and the number of CFU was determined.

Isolation and culture of mouse tracheal epithelial cells. To study the effect of M. pulmonis on epithelial electrolyte transport, mouse tracheal epithelial cell cultures were prepared as previously described (51, 52). Animals were sacrificed by CO2 inhalation and opened surgically with a ventral midline incision. The intubation trachea was removed from the bifurcation. The intact trachea was stripped of remaining connective tissue and cut longitudinally to expose the epithelial cells. Tissues were washed two times with minimal essential medium (MEM; Gibco/BRL, Grand Island, N.Y.) and placed in solution A (MEM, 1% fetal bovine serum [HyClone Laboratories], 0.1% protease XIV [Sigma Chemical Co., St. Louis, Mo.], 8 U of DNase [Sigma]) at 4°C for 16 to 24 h with intermittent vortexing. Cells were recovered by vigorously vortexing the tracheas in solution A followed by centrifugation at 500 g for 5 min. The pellet cells were resuspended in MTE medium, which was made by mixing equal volumes of preconditioned medium (Dubelco’s MEM [Gibco/BRL], 10% fetal bovine serum [recollected after feeding a confluent monolayer of 3T3 fibroblasts for 2 days], 1% Pen/Strep [Gibco/BRL] and solution B (Ham’s F-12 medium [Gibco/BRL] with 1 μg of insulin per ml, 7.5 μg of transferrin per ml, 1 μM hydrocortisone, 30 nM 3,3′,5-triiodo-L-thyronine sodium, 1 ng of chola toxin per ml, 2.5 ng of epidermal growth factor per ml, and 10 ng of endothelial cell growth substance per ml [all purchased from Sigma]). The cell suspension was seeded onto porous 0.4-μm-pore-size Transwell support filters (Corning Costar, Cambridge, Mass.) that had been previously coated with type VI collagen (Sigma). MTE medium was added to the outside chamber of each filter, and the filters were incubated at 37°C in the presence of 5% CO2 to be equilibrated with 65 ml of medium per filter in three times per week for 2 to 3 weeks. The monolayer confluency was monitored by measuring the ability of the cells to hold back culture medium from their apical surface and by unidirectional [3H]mannitol flux experiments (4, 5). Mono-

layered cultured monolayers were used for these experiments. A freshly thawed stock of M. pulmonis was diluted in MTE-conditioned medium. Epithelial cells were apically inoculated with 125 μl of medium that contained a total of 0.5 × 10^5 to 5.0 × 10^7 CFU of M. pulmonis (4 × 10^6 to 4 × 10^7 CFU/ml) for basolateral infection experiments, the basolateral surface of the filters was exposed to 500 μl of medium which contained between 2 × 10^5 and 2 × 10^6 CFU of M. pulmonis (4 × 10^4 to 4 × 10^5 CFU/ml). Control filters were inoculated with sterile mycplasma culture medium diluted in MTE-conditioned medium at the same ratio. Minion et al. have previously reported that a substantial number of M. pulmonis cells become cell associated within an hour after infection (25). Furthermore, we have also determined that mycoplasma adherence to eukaryotic cells does not increase significantly after 6 to 8 h (unpublished results). As a result of these findings, monolayers were inoculated with mycoplasma culture medium or control medium and incubated at 37°C in the presence of 5% CO2 for 6 h unless otherwise described. After incubation, control and mycoplasma-infected monolayers were washed two times with MTE-conditioned medium (125 μl) and then incubated at an air interface for an additional 42 h prior to use in Ussing chambers. Forty-eight hours postinoculation was chosen as the main time point to evaluate transport on the basis of previous studies by Städtlander et al. (39) which showed that an M. pulmonis inoculum of 10^5 to 10^7 cells resulted in comparable expression of organ culture values. In the time course experiments, monolayers were similarly exposed to the control or the mycoplasma inoculum for 6 h, washed, and then incubated at an air interface for an additional 6 or 12 h. We noted that the filters assayed at either 12 or 18 h postinoculation yielded similar electrolyte values than those assayed at 48 h. We determined that this decrease was due to the continued presence of the medium on the apical surface of the monolayers prior to their use and that this effect could be reversed if the monolayers were incubated at an air interface for an additional 12 to 24 h. Widdicombe et al. (50) recently reported that the continued presence of culture medium on the apical surface of human tracheal epithelial cells also caused a reduction in transepithelial potentials. The result of these findings, monolayers were inoculated with mycoplasma-containing inocula that had not been exposed to UV light. The following experimental protocol was utilized to assess the Na+- and Cl– secretory capacity of the uninfected (control) and M. pulmonis-infected monolayers. Under standard culture conditions, the mouse respiratory epithelial monolayers are inherently Na+- absorptive. When the MTE filter was mounted in the Ussing chamber, the magnitude of transepithelial Na+ current was determined by adding amiloride (10 μM) to the mucosal bathing solution. The current inhibited by amiloride is referred to hereafter as the amiloride-sensitive Na+ current (I Na,AM). After 10 to 15 min, forskolin (10 μM) was added to the apical and basolateral surfaces of the monolayers. Each transmural current was divided into 1-ml aliquots and stored at −70°C. Quantitation of mycoplasmas and then incubated for an additional 42 h at 37°C. The filters were then prefixed with 1.5% glutaraldehyde for 90 min at 4°C, washed two times with phosphate-buffered saline prior to the secondary fixation with 1% osmium tetroxide at room temperature. Dehydration was performed for a graded series of ethanol-water solutions, and specimens were transferred to propylene oxide and embedded in epoxy resin. Ussing sections were cut and stained by standard methods and examined as previously described (39).

Statistical analysis. Results are presented as the means ± standard errors of the means (SEMs). Effects attributable to experimental manipulations were assessed for statistical significance by employing Student’s t test for paired and unpaired data, as appropriate. Experimental treatment effects were considered statistically significant if the probability of a type I error was <0.05 or otherwise noted.
RESULTS

Inhibition of epithelial ion transport by *M. pulmonis*. Strikingly different $I_{sc}$ profiles were observed when control (mock-infected) and *M. pulmonis*-infected MTEs were evaluated for $Na^+$ absorption and $Cl^-$ secretion in Ussing chambers (Fig. 1). Prior to the addition of amiloride, the control MTE monolayer mounted in Ussing chambers displayed a resistance of $2,336 \, \Omega/cm^2$ and an $I_{sc}$ across the monolayer of $27.0 \, \mu A/cm^2$ (Fig. 1A). The addition of amiloride to the apical buffer rapidly reduced the $I_{sc}$ to $7.1 \, \mu A/cm^2$, resulting in the identification of an $I_{Na^+}$ of $19.9 \, \mu A/cm^2$. Forskolin caused an increase in the $I_{sc}$ of $11.4 \, \mu A/cm^2$. Carbachol, a $Ca^{2+}$-mediated agonist, rapidly increased the $I_{sc}$ to a peak value of $78.6 \, \mu A/cm^2$. Subtraction of the $I_{Na^+}$ from this total increase resulted in an $I_{Cl_{cAMP}}$ peak of $67.2 \, \mu A/cm^2$. The $I_{Cl_{peakAS}}$ was similarly calculated and found to be $38.6 \, \mu A/cm^2$. After an additional 10 to 15 min, the addition of furosemide to the basolateral solution reduced the $I_{sc}$ to $21.4 \, \mu A/cm^2$, verifying that the $I_{sc}$ was due to net $Cl^-$ secretion. MTE monolayers that had been infected with *M. pulmonis* were similarly evaluated and demonstrated a dramatic decrease in transepithelial ion transport (Fig. 1A and D). After monolayers were infected with an initial inoculum of $1.9 \times 10^7 \, CFU$ (Fig. 1D), the $I_{Na^+}$ was $1.8 \, \mu A/cm^2$, and the $I_{Cl_{cAMP}}, I_{Cl_{peak}},$ and $I_{Cl_{peakAS}}$ values were 4.29, 11.4, and 6.8 $\mu A/cm^2$, respectively. The results of 13 to 16 additional experiments are summarized in Table 1. In addition, the transepithelial resistance of infected monolayers was also substantially altered. These monolayers displayed a resistance of only $440 \pm 87 \, \Omega/cm^2$ (mean ± SEM). Unidirectional $[^3H]$mannitol flux studies revealed a 2.1-fold-higher flux across *M. pulmonis*-inoculated monolayers (2.3 ± 0.33 nmol, $n = 4$) than across control monolayers (1.1 ± 0.21 nmol, $n = 4$) ($P < 0.01$). These results are consistent with the 5.4-fold-lower resistance of the *M. pulmonis*-infected monolayers described above and confirm that *M. pulmonis* significantly decreases the paracellular resistance of the MTE monolayers. In contrast to the results described above in which the apical surface was infected, paired experiments in which the basolateral surface of the MTEs was exposed to either the control medium or the mycoplasma inoculum ($2 \times 10^7$ to $2 \times 10^8$...
CFU, (n = 6) were also performed. The $I_\text{sc}$ values for mono-
layers exposed basolaterally to the control inoculum included an $I_\text{Na}^+$ of 23.4 ± 2.8 μA/cm², an $I_{\text{Cl}\text{-cAMP}}$ of 11.9 ± 1.5 μA/cm², and an $I_{\text{Cl}\text{-peak}}$ of 108.0 ± 12.5 μA/cm² at 48 h. Prior to the assessment of the transport capacity of the MTE filters that had been basolaterally infected with M. pulmonis, the basolateral feeding medium was cultured to determine the number of CUC of M. pulmonis present. Approximately 10⁶ CUC of M. pulmonis were routinely recovered from the baso-
laterally infected filters which displayed an $I_\text{sc}$ of 24.5 ± 1.7 μA/cm², an $I_{\text{Cl}\text{-cAMP}}$ of 11.5 ± 1.6 μA/cm², and an $I_{\text{Cl}\text{-peak}}$ of 118.8 ± 8.2 μA/cm². These results indicate that exposure of the basolateral surface of the monolayer of M. pulmonis did not alter electrogenic ion transport.

Altered transport following exposure of the apical surface to M. pulmonis was not merely a result of epithelial cell death, as evidenced by the following: (i) maintenance of a mean transepithelial resistance of 440 Ω·cm², which is 20 times greater than fluid resistance; (ii) lack of leakage of fluid from the basolateral to the apical surface; and (iii) normal cell morphology, including intact tight junctions, as demonstrated by electron microscopy performed at the time of altered ion transport. Transmission electron micrographs of control MTEs revealed a confluent monolayer of uniform, columnar epithelial cells characterized by tight junctions, microvilli, and a highly con-
voluted basolateral membrane (data not shown). These char-
acteristics are consistent with the high electrical resistance measured in the Ussing chambers. MTEs examined as late as 48 h after M. pulmonis infection were morphologically identical to the control monolayers (data not shown). Mycoplasmas were not observed in association with the epithelial cells; how-
ever, quantitative cultures of infected monolayers documented that the organisms were not only present but also replicating (Table 2). Since the number of epithelial cells comprising the confluent monolayer was estimated to be $3.0 \times 10^5$, at 48 h postinoculation, the number of mycoplasmas per cell (i.e., approxi-
mately 17) may not be easily detectable by electron mi-
croscopy.

**Effect of organism number and duration of exposure.** Increasing the number of M. pulmonis cells in the infecting in-
oculum resulted in a dose-dependent decrease in both ion transport and transepithelial resistance at 48 h (Fig. 1). The length of exposure of the monolayer to the M. pulmonis inocu-
ulum was also varied so that shorter incubation times could be evaluated. Monolayers that were inoculated with M. pulmonis for 1, 3, or 6 h, washed, and immediately assayed displayed a dramatic time-dependent decline in their $I_\text{sc}$ profile compared to that of the control filters (Fig. 2). The monolayers exposed for 6 h displayed a mean transepithelial resistance of only 53 Ω·cm², whereas the controls maintained a resistance of 1,571 Ω·cm². In addition, the infected monolayers did not display $\text{Na}^+$ transport and no longer responded to pharmacological stimulators of Cl⁻ secretion. Interestingly, 6-h-infected MTEs that were washed and allowed to recover for an additional 18 h displayed resistance values of $1,434 \pm 439$ Ω·cm² (n = 10). These values were similar to those of the control MTEs of $1,746 \pm 312$ Ω·cm² (n = 14) (Fig. 2).

**Viable organisms are required for inhibition of ion transport.** Experiments were performed to determine if inhibition of electrolyte transport was due to a substance released by the mycoplasmas into the medium in which they were grown. MTEs were exposed for 6 h to mycoplasma culture superna-
tants or uninoculated culture medium. The monolayers were washed and then incubated for an additional 42 h. No differ-
ces in $I_\text{sc}$ values were observed (Fig. 3A). To determine if mycoplasma-conditioned culture medium had an acute affect on ion transport, MTEs were exposed to culture supernatants for 1, 3, and 6 h, washed, and immediately assayed. The ion-
transporting capacity of these acutely exposed monolayers was also not statistically different from that of the control mono-
layers exposed to uninoculated mycoplasma medium for the same intervals (n = 3) (data not shown). These results indicate that altered transport requires interaction with an organism. To determine whether organism viability is also required for alteration of ion transport, MTEs were exposed to either the control or the M. pulmonis inoculum which had been UV irradiated. There was no statistical difference between the ep-
ithelial ion transport capacity and the transepithelial resistance of MTEs infected with the UV-irradiated M. pulmonis inocu-
ulum (<1% viable organisms remaining) and of those exposed to UV-irradiated uninoculated medium (Fig. 3B). These values were also not different from those of MTE monolayers exposed to unirradiated control inocula (data not shown).

**Nonmurine mycoplasma does not affect ion transport.** To determine if the effects of the M. pulmonis inoculum were mycoplasma species specific, a nonmurine mycoplasma was evaluated for its ability to alter transepithelial ion transport by using conditions identical to those described above for M. pul-
monis. M. fermentans, a mycoplasma of human origin, was used to inoculate MTEs in numbers ranging from $5 \times 10^3$ to 60-fold greater than those used for M. pulmonis. M. fermentans had no effect on epithelial transport, despite the recovery of large numbers of M. fermentans from infected filters at 48 h (Table 2). The transepithelial resistance of the M. fermentans-
inoculated monolayers was also not significantly different from the controls.

**Table 1. Effect of mycoplasma on MTE epithelial ion transport**

<table>
<thead>
<tr>
<th>Inoculum (no. of expts)</th>
<th>$I_{\text{sc}}$ value (μA/cm²)</th>
<th>Resistance (Ω·cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hayflick’s medium (control) (n = 16)</td>
<td>35.1 ± 2.8</td>
<td>8.42 ± 1.1</td>
</tr>
<tr>
<td>M. pulmonis CT (n = 13)</td>
<td>8.7 ± 1.6⁷</td>
<td>4.4 ± 0.7⁸</td>
</tr>
<tr>
<td>SP4 medium (control) (n = 4)</td>
<td>43.1 ± 5.7</td>
<td>21.9 ± 4.3</td>
</tr>
</tbody>
</table>
| M. fermentans incon- 
nitrus (n = 6) | 34.2 ± 3.8 | 17.7 ± 2.0 | 92.2 ± 22.6 | 1,064 ± 264 |

* MTEs were inoculated for 6 h, washed, incubated for an additional 42 h, and assayed for epithelial ion transport as described in Materials and Methods. The data represent the means ± SEMs.

⁷ Significantly different from value for control at P of <0.01.

⁸ Significantly different from value for control at P of <0.001.

**Table 2. CFU recovered from MTEs after infection with mycoplasma**

<table>
<thead>
<tr>
<th>Time (h) postinfection</th>
<th>M. pulmonis CT (n = 4)</th>
<th>M. fermentans incongritus (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>(3.0 ± 0.01) × 10⁴</td>
<td>ND</td>
</tr>
<tr>
<td>48</td>
<td>(5.1 ± 1.1) × 10⁴</td>
<td>(8.2 ± 2.9) × 10⁵ ND</td>
</tr>
<tr>
<td>72</td>
<td>(8.6 ± 3.5) × 10⁵</td>
<td>ND</td>
</tr>
<tr>
<td>96</td>
<td>(1.2 ± 1.9) × 10⁵</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Values are means ± standard errors for numbers of CFU recovered from infected filters 48 h postinfection. ND, not determined. n, numbers of expe-
riments.
**DISCUSSION**

Previous in vitro studies have indicated that *M. pulmonis* can attach to eukaryotic cells within 1 h of exposure (25). The results presented here provide evidence that upon interaction with the respiratory epithelium, *M. pulmonis* may rapidly alter the electrolyte composition of the fluid layer which lines the airways of its natural host. A 1-h exposure of MTEs to *M. pulmonis* resulted in a substantial decrease in its ion-transporting capacity and its electrical resistance. As the length of exposure to the inoculum was increased, both transport functions continued to decrease. MTEs inoculated with *M. pulmonis* for 6 h and then immediately assayed had lost all detectable transport function and almost all transepithelial resistance. Those that were exposed to the inoculum for 6 h, washed, and then incubated for an additional 18 h recovered all of their transepithelial resistance and some of their active-transport capacity. Thus, a 6-h exposure severely compromised the barrier integrity and transport capacity but did not kill the epithelial cells. Several explanations may account for the observed recovery of the monolayer resistance as well as the partial recovery of active transport. The first suggests that the number of organisms associated with the monolayer directly influences its transport capacity. Washing the monolayer after a 6-h exposure to the inoculum may have removed most of the *M. pulmonis* cells unassociated with the monolayer, thereby allowing it to recover. Forty-eight hours after inoculation, another significant decrease in the resistance of the monolayers occurred. This was again consistent with a measured increase in the number

![Figure 2: Acute time-dependent effects of *M. pulmonis* on transepithelial ion transport and monolayer resistance. A representative series of traces of the $I_{sc}$ profile at 1, 3, 6, or 24 h after exposure of the MTE monolayers to mycoplasma medium (control) or *M. pulmonis* ($2.0 \times 10^7$ CFU) is shown. It is important to note the time-dependent decrease in monolayer resistance over 1 to 6 h and the recovery at 24 h. The inhibition of $I_{Na}$, $I_{CT-cAMP}$, and $I_{CT-peak}$ were also time dependent but appeared to only partially recover by 24 h.](http://iai.asm.org/Downloaded from)
of *M. pulmonis* cells associated with the monolayer. A second explanation suggests the intriguing possibility that the interaction of *M. pulmonis* with the monolayer may cause a change in the population of mycoplasma cells. Adherence to the monolayer could induce or select for a less cytopathic population of organisms, allowing the epithelium to survive and reestablish ion transport but at a much reduced level. In these studies, the switching of the organisms from liquid culture media to an air-epithelium interface could trigger a phenotypic change in the cell-associated population. This explanation is consistent with previous studies that have shown that in infected animals *M. pulmonis* undergoes both phenotypic and genotypic changes which are associated with chronicity (37, 45). In contrast to the transepithelial resistance, when the monolayer’s capacity for Na\(^+\) absorption or Cl\(^-\) secretion was inhibited, transport of neither pathway returned to control levels. These results suggest that the decrease in transepithelial resistance and the inhibition of electrogenic ion transport by *M. pulmonis* may be independently mediated.

Together, these studies indicate that the addition of *M. pulmonis* to the apical surface of MTEs rapidly alters both epithelial barrier integrity and transport function. The failure of *M. pulmonis* to affect ion transport from the basolateral side reinforces the importance of interaction with the apical surface and is consistent with in vivo studies of mice which suggest that *M. pulmonis* remains limited to the apical surface, i.e., it is rarely found in the submucosa (39). The epithelial cells were grown on a 0.4-μm-pore-size filter, and it is possible that the filter precluded the interaction of the mycoplasmas with the basolateral cell surface despite their small cellular size (180 to 300 nm) and their plasticity associated with the lack of a cell wall (44). *M. pulmonis* cells (1 × 10^6/ml) were recovered from the basolateral medium after a 48-h incubation, indicating that their presence on the basolateral side was insufficient to alter transport. We were unsuccessful in our attempts to grow MTEs on a more porous support (4.0-μm pore size) to test further whether MTE-*M. pulmonis* contact at the basolateral membrane would alter transport.

Other investigators have previously shown that *M. fermentans*, a mycoplasma of human origin, does not cause cytopathic effects in mice following intranasal inoculation or in murine tracheal organ cultures (40). Consistent with these results, ion transport was also not altered when the MTE monolayers were infected with *M. fermentans*, despite the large numbers of *M. fermentans* cells recovered from the monolayers at 48 h postinfection. Thus, the presence of mycoplasma cells per se does not lead to the alterations observed in the MTEs but rather suggests that a species-specific interaction between *M. pulmonis* and the MTE monolayer would seem to be required. To verify this, additional species of mycoplasma must be studied in the MTE monolayer system.

Although several potential pathogenic factors have been identified in the mycoplasmas, including peroxidases (10), endonucleases (30, 32), phospholipases (16, 34), and a membrane-associated hemolytic activity (10, 27), the molecular mechanisms by which *M. pulmonis* colonizes the respiratory epithelium and establishes chronic airway disease are unknown. Exposure of the monolayers to *M. pulmonis* culture supernatants did not alter Na\(^+\) absorption, Cl\(^-\) secretion, or the resistance of the MTEs. These results indicate that the *M. pulmonis* component responsible for the alterations in electrogenic ion transport and the decrease in epithelial resistance of the MTE monolayer is not released by *M. pulmonis* cells under normal laboratory culture conditions but requires the intact organism. Additional studies will be needed, however, to determine if the inhibiting activity is selectively secreted by

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**FIG. 3.** Lack of effect of *M. pulmonis* culture supernatant or UV-irradiated *M. pulmonis* cells on epithelial ion transport. (A) Monolayers were incubated with either sterile mycoplasma medium (control) or filtered (0.1-μm pore size) supernatant obtained from *M. pulmonis* stocks for 6 h. Subsequently, the monolayers were washed twice with MTE conditioned medium and incubated for an additional 48 h prior to evaluation in Ussing chambers. Results are the mean *I*_sc values obtained from five experiments ± SEM. (B) Lack of effect of UV-irradiated *M. pulmonis* inoculum for 6 h. After incubation, the monolayers were washed twice with MTE conditioned medium and incubated for an additional 42 h in Ussing chambers prior to evaluation: Results are the mean *I*_sc values obtained from three experiments ± SEM.
mycoplasma cells upon contact with the epithelium. Several studies have examined the pathogenic potential of purified mycoplasma membranes, cell lysates, and nonviable organisms (2, 12, 19, 26). In our studies, M. pulmonis cells that were killed by UV treatment were no longer able to inhibit the absorption or secretion of ions or to decrease the transepithelial resistance of the monolayer.

Numerous researchers have reported that the infection of tracheal organ cultures with mycoplasmas results in ciliostasis, loss of tight junctions, and in most cases the complete exfoliation of the respiratory epithelium (2, 9, 11, 15, 24, 39). This study, in contrast, evaluated the electrogenic ion transport capacity of MTEs infected with M. pulmonis and reveals that this organism causes much more subtle changes in the respiratory epithelium than have previously been observed by other investigators. The inhibition of Na\(^+\) absorption and Cl\(^-\) secretion and the concomitant loss in resistance of the epithelial monolayer suggest that M. pulmonis infection leads to the formation of a new functional steady state in which the monolayer continues to absorb and secrete fluid and electrolytes but at a much reduced level. The pathophysiological consequences of this reduced functional state are a change in the volume and composition of the fluid which bathes the airways. This could, in turn, compromise ciliary movement and mucus hydration and ultimately lead to a reduction in mucociliary clearance.

Ion transport across the epithelial monolayer is a highly complex and tightly regulated molecular cross talk between extracellular signals, intracellular second messengers, and the transport proteins in the apical and basolateral cell membranes. Therefore, the modulation of ion transport and transepithelial resistance by M. pulmonis could occur at a variety of sites. The absorption and secretion of fluid and electrolytes across the respiratory epithelium involve the coordinated movement of ions across the apical and basolateral membranes through highly selective Na\(^+\), Cl\(^-\), and K\(^+\) channels, the Na\(^+\)-K\(^+\) pump, and the Na\(^+\)-2Cl\(^-\) cotransporter, any of which could be directly or indirectly altered during mycoplasmal infection. The ability of M. pulmonis to alter host cell second messengers is a logical explanation for the observed decrease in both Na\(^+\) and Cl\(^-\) transport; however, the factors mediating these alterations have yet to be clearly addressed for any mycoplasma species (36). Both Na\(^+\) and Cl\(^-\) were inhibited in our studies, suggesting that M. pulmonis may alter a single step which is central to both pathways (e.g., the basolateral K\(^+\) channel). This notion is supported by the recent studies of Izutsu et al., who showed that Mycoplasma orale infection affects the expression and regulation of K\(^+\) channels in the surface membrane of a human submandibular gland cell line (22).

Recent studies by Smith et al. (38) indicate that pathogenic microorganisms may persist in the airways of patients with cystic fibrosis because bacteriocidal factors normally produced by the respiratory epithelium are inactivated by abnormal salt concentrations. Our results suggest that the ability of M. pulmonis to alter transepithelial ion transport may allow it to escape killing by a similar mechanism. This is further supported by the fact that M. pulmonis-infected animals are incapable of clearing the organism from the respiratory epithelium and are also highly susceptible to secondary infections. It is safe to assume that the pathophysiology which underlies chronic mycoplasmal infections is undoubtedly complex and multifactorial in origin. The methodologies described herein may be useful in determining which pathogenic factors are involved and, in turn, guide us toward the most logical approach for therapy against persistent mycoplasmal diseases.

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