Oxygen Consumption by Trachea Organ Cultures Infected with *Mycoplasma pneumoniae*

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Hamster trachea organ cultures were shown to consume measurable amounts of oxygen when incubated in a closed micro-chamber. Several simple and complex media were evaluated, and the optimal system involved incubating 2 to 4 mg of tracheal tissue in Tyrodes balanced salt solution at 37 C for 60 min, with oxygen activity measured with a Clark electrode. Trachea infected with *Mycoplasma pneumoniae* showed a significantly lower oxygen utilization than did uninfected controls. The effect was dose dependent when levels of 10^6 to 10^7 colony-forming units per ml were used to infect the tracheal rings. Virulent mycoplasmas caused significant decreases in relative ciliary activity and oxygen utilization, whereas attenuated mycoplasmas reduced ciliary activity and oxygen utilization levels to a lesser extent.

The pathogenesis of *Mycoplasma pneumoniae* infections can be studied conveniently in hamster trachea organ cultures (3, 4, 7). This model system alleviates one of the major drawbacks encountered in other organ cultures, i.e., the difficulty in obtaining continuous measurements of cellular activity, because one can visually observe the beating of the cilia on the epithelial side of the trachea explant or slice. Estimates of necrosis and ciliary activity can be based on the percentage of the tissue remaining intact (7), as well as the presence (4), vigor (2, 7), or frequency of ciliary motion (3). Recently, methodology has been introduced whereby protein and ribonucleic acid synthesis can serve as an index of metabolic activity in infected tissue (11).

During our work on the mechanism of pathogenesis in mycoplasma pneumonia, we measured the oxygen utilization by ciliated respiratory epithelium in trachea organ cultures and found marked differences when control and infected cultures were compared. The investigation described here concerns the development of the assay and its application to the study of host-parasite interactions in infections with *M. pneumoniae*.

**MATERIALS AND METHODS**

**Mycoplasmas.** *M. pneumoniae*, strain FH, was provided by J. Tully (National Institutes of Health), and strains Mac (attenuated) and PI 104166 (virulent) were furnished by W. Clyde (University of North Carolina). Organisms were grown in SSRL broth (13), and log-phase cells were collected by centrifugation at 12,000 × g for 45 min. Cells were resuspended in organ culture medium, and appropriate dilutions were made. Actual cell counts were based on serial 10-fold dilutions plated in duplicate on Hayflick agar and incubated for 7 to 14 days before counting.

**Organ culture medium.** Trachea organ cultures were maintained in 90% Eagle minimal essential medium (MEM) and 10% fetal calf serum, supplemented with L-glutamine (40 mM), penicillin G (200 U/ml), and N-2-hydroxyethyl-piperazine-N’-2’-ethane sulfonic acid (25 mM).

**Organ cultures.** Tracheas from adult Golden hamsters were prepared as described previously (7). Cultures were inoculated on day 0, and relative activity measurements, i.e., percentage of rings intact (0 to 100) multiplied by the vigor of ciliary beating (0 to 4), were made daily during observations with phase optics (×225).

**Incubation solutions.** Ringer solution (6), Tyrodes balanced salt solution (10), and modified organ culture medium, MEM/SF (same as organ culture formula, but with 1% serum fraction instead of 10% fetal calf serum; plus 1% glucose), were evaluated.

**Measurements of respiratory activity.** After 4 to 6 days in culture (with or without mycoplasmas), tracheal rings were rinsed four times in phosphate-buffered saline, pH 7.4 (7) and were placed on the plunger of a sterile, 1.0-ml disposable glass syringe (Fig. 1). This was carefully slid into the syringe barrel until the edge was at the 0.2-ml mark. The incubation medium, prewarmed and sparged with air or oxygen, was contained in a 10-ml syringe with a 20-gauge needle. Medium was injected into the 1.0-ml syringe (containing the tissue) through its tip. The syringe with tissue and 0.2 ml of medium was then capped with a 0.5-inch (about 1.25 cm), 26-gauge needle. Air was expelled by pushing the plunger down to the 0.1-ml mark, and the needle was sealed with clay (Critoseal, Sargent Welch, Chicago, Ill.).

The sealed syringes containing tissue were placed
PREPARE EXPLANTS

MEASURE ACTIVITY

Fig. 1. Diagrammatic representation of the process used to prepare tissue samples for measurement of oxygen consumption with the aid of a blood gas analyzer.

in a roller drum (2 rpm) and were incubated for 60 min at 37°C. The "tumbling" of the tracheal rings served to agitate the fluid and thereby prevent gradients from forming. To measure oxygen activity, the needle was removed and a 100-ml sample was aspirated into the measuring chamber of a blood gas analyzer (IL 213, Instrumentation Laboratory, Inc., Lexington, Mass.). A Clark oxygen electrode (9) in the chamber measured activity and provided the pO₂ (in millimeters) in a digital readout. Daily electrode standardization was conducted with 0.0 and 12.0% O₂ (IL certified gases). Equilibration occurred within 120 s.

Values from a blank (no tissue) sample served as the reference point, and oxygen utilization by control or infected tracheas was developed as the ΔpO₂ (in millimeters), i.e., blank value minus the sample value. Assays were normally done in triplicate. Trachea rings were weighed in order to calculate the Q(O₂) value, (ΔpO₂ in millimeters per milligram [wet weight] per 1-h incubation period). Infected tissue was always compared with its control run simultaneously.

RESULTS

Several solutions were evaluated in order to determine the optimal incubation medium. A simple basic salt solution (Ringer), a complex salt solution containing glucose (Tyrode), a serum-fraction broth (MEM/SF), and a broth containing fetal calf serum (MEM) were compared for their ability to support healthy tissue. Each could maintain viability and permit measurable depletions in oxygen activity of 17 to 21% of the blank value when starting with air-saturated solutions (Table 1).

Similar results were noted when the solutions were sparged with 95% oxygen. Though considerable oxygen loss occurred during handling and preparation, final values were in excess of 200 mm (Hg) of pressure. This resulted in somewhat greater oxygen utilization than that seen with air-sparged solutions. Tyrode solution consistently gave high blank O₂ values (approximately 250 mm) with nearly 50-mm deflections with trachea rings and had minimal standard deviations (249 ± 6 mm), so it was chosen as the incubation medium for subsequent studies.

The optimal amount of tissue per chamber was determined by incubating one to six trachea rings in sealed syringes and measuring the drop in pO₂ after 60 min of incubation. The results from 29 separate determinations are given in Fig. 2. Oxygen utilization increased in a regular fashion with larger amounts of tissue, but when more than 4 mg was used, no significant increase in oxygen depletion was detectable. Experiments with infected tissue were thus conducted with two to three rings per 0.1-ml chamber to obtain the most efficient system.

To determine whether trachea explants infected with mycoplasma would show altered oxygen consumption, several trachea rings were infected with approximately 10⁸ colony-forming units (CFU) of M. pneumoniae, strain FH (Fig. 3). Relative activity counts were made on days 0 to 6, and the O₂ uptake was measured after the last reading. Mean data from five control and five infected dishes with three rings each show that controls maintained reasonably vigorous activity (i.e., approximately 300), whereas the infected cultures dropped rapidly after 24 h, until the relative activity was less than 50 on day 5. Control cultures consumed oxygen at a rate of approximately 32 mm/mg per h, whereas tracheas exposed to M. pneumoniae and showing obvious necrosis and ciliostasis consumed about half as much oxygen (18 mm/mg per h).

The measurable differences in oxygen utilization by control and infected trachea rings prompted an examination of the dose-dependency of the phenomenon. Separate cultures were exposed to either 10⁴, 10⁵, or 10⁶ CFU of M. pneumoniae FH per ml, and values of relative ciliary activity and oxygen consumption were measured. Figure 4, containing mean data from three experiments (total of 27 rings for each point), shows how the drop in ciliary activity was dose dependent and directly related to the dose of viable mycoplasmas.

On day 5, oxygen utilization by these rings was determined and compared with the relative activity data (Fig. 5). It was noted that the ΔpO₂ (i.e., change in oxygen activity per 0.1-ml chamber with two to three rings) and Q(O₂) (ΔpO₂ in millimeters per milligram per hour) were lowest with the 10⁶-CFU dose. However, the results with 10⁵ CFU were essentially the
Table 1. Influence of incubation medium on oxygen utilization by trachea organ cultures

<table>
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<tr>
<th>Medium</th>
<th>Tissue</th>
<th>Wt (mg)</th>
<th>$pO_2$ (mm)</th>
<th>$\Delta pO_2$ (mm)</th>
<th>% Change</th>
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</thead>
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<tr>
<td>Tyrode BSS*</td>
<td>Blank</td>
<td>0</td>
<td>165.8 ± 4.0</td>
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<td>—</td>
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<tr>
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<td>Trachea</td>
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<td>137.8 ± 12.0</td>
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<td>16.9</td>
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<tr>
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<td>163.0 ± 2.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>Trachea</td>
<td>1.3</td>
<td>132.4 ± 7.2</td>
<td>30.6</td>
<td>18.8</td>
</tr>
<tr>
<td>MEM/SF</td>
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<td>156.4 ± 3.3</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<td>113.5 ± 18.4</td>
<td>32.4</td>
<td>21.1</td>
</tr>
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</table>

* Each value represents mean data (± standard deviation) from five separate tracheal rings incubated for 60 min in 0.1-ml chambers. Media formulations are given in the text.

* BSS, Balanced salt solution.

Fig. 2. Effect of the amount of tracheal tissue on oxygen utilization in a 0.1-ml chamber incubated for 60 min at 37 C.

Fig. 3. Effect of infection with M. pneumoniae on relative ciliary activity and oxygen uptake by trachea organ cultures. Each point and bar represents mean data from 15 rings (+ 1 standard deviation).

Fig. 4. Dose-response relationship of number of infecting mycoplasmas (M. pneumoniae FH) and relative ciliary activity in trachea organ cultures versus time.

Contains mean data from four separate determinations on uninfected tissue and on rings exposed to $4 \times 10^4$ CFU of M. pneumoniae Mac (attenuated) or $10^6$ CFU of M. pneumoniae PI 104166 (virulent). Readings of relative ciliary activity for uninoculated controls remained quite high, indicating an intact, vigorously heating ciliated epithelium. This was confirmed in the $Q(O_2)$ value. The virulent M. pneumoniae (PI 104166) rapidly induced ciliostasis, with a resultant relative activity of less than 50 after 4 days. This damage was also reflected in the $Q(O_2)$ value, which was noticeably lower than that of the uninfected samples (49, as compared with 65 for the controls).

The relatively avirulent strain of M. pneumoniae (Mac) caused a much smaller reduction in the ciliary activity, even though the inoculum was higher than that used with the virulent strain (Fig. 6). The intermediate nature of the effect on ciliary motion was also noted in the oxygen consumption rate, where the value of 56 mm/mg per h was at the low end of the standard error zone for the uninfected controls.
and at the high end of the standard error margin for tracheas exposed to virulent *M. pneumoniae*.

**DISCUSSION**

The application of trachea organ cultures (2-4, 7) to the study of mycoplasma-induced disease processes results in obvious advantages over in vivo systems. As with most organ cultures, trachea explants eliminate or minimize hormonal effects and immunological responses, as well as age, weight, and sex differences. In addition, the number of cells or quantity of toxin necessary to elicit an effect is normally quite small.

One of the main attributes of the trachea culture as an infectious disease model is the relative ease with which metabolic changes can be noted. The system presented here, based on the ability of trachea explants to utilize measurable amounts of oxygen in a closed system, exploits this potential. Small amounts of tissue (less than 4 mg) could be incubated in oxygenated Tyrode solution contained in 0.1-ml micro-chamber. Values of oxygen activity (15), directly related to but not identical with oxygen concentration, were measured by a Clark electrode. Values of respiratory activity obtained from uninfected tissue were similar to those reported for rat fetal heart (16).

It should be noted that oxygen uptake can be measured in relative units (12), microliters (14), micrograms (1), or millimeters of pressure (16). Any of these can be used when making comparisons between control and treated tissue. The latter was chosen here because of its simplicity and to facilitate application of the method for use with a variety of blood gas analyzers and oxygen electrodes. If necessary, standardizations (5, 15) can be performed to interconvert the various parameters. Such a procedure is recommended for each buffer system to accommodate changes in oxygen solubility resulting from differences in molarity, though such solubility differences are usually less than 10% (5, 10).

When trachea explants were infected with *M. pneumoniae*, relative activity of the cilia steadily decreased with time. After 5 days in culture, such infected explants used about half as much oxygen as uninfected controls. It is unlikely that the change was an indirect result of mycoplasma growth in the culture fluid since mycoplasmas do not multiply in the organ culture medium (apparently due to the lack of horse serum and yeast extract). The altered respiration we observed is consistent with a recent report (11) indicating that protein and ribonucleic acid synthesis, as well as carbohydrate utilization, is significantly depressed in trachea organ cultures 48 h after infection with virulent mycoplasmas.

When the dose of *M. pneumoniae* was varied, it was noted that the ciliary response and respiration changes were related, i.e., high doses (10⁶ CFU) resulted in the lowest activity of both \(Q(O_2)\) and relative activity. However, it was a consistent finding that moderate doses (10⁴ CFU) resulted in respiration activity at about the control level, even though ciliary activity

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**Fig. 5.** Comparison of ciliary activity (relative activity) and oxygen uptake (\(\Delta pO_2\) and \(Q(O_2)\)) of tracheas exposed to 0, 10⁵, 10⁶, or 10⁷ CFU of *M. pneumoniae* FH per ml.
was lowered by more than 50%. This may be an indication that a microbial load of that size can cause some moderate necrosis that is accompanied by cellular regeneration or repair, and/or increased metabolic activity by the intact epithelium or mitochondria (1).

The relationship of intactness of the ciliated epithelium and pronounced cellular respiration was further corroborated by data obtained with virulent and avirulent mycoplasmas. High doses of the attenuated *M. pneumoniae*, strain Mac, gave ciliary activity values intermediate to those of the uninfected controls and tracheas exposed to virulent (PI 104166) *M. pneumoniae*. The Q(O₂) (milliliters per milligram per hour) from such tracheas was similarly intermediate to the respiratory quotients from other types of tissue.

It is unlikely that the oxygen utilization detected by this technique was due to mycoplasma cells because of the relatively few numbers of mycoplasma cells on the trachea rings. Our data (submitted for publication) indicate that it requires at least 10⁴ CFU of *M. pneumoniae* or *Acholeplasma laidlawii* per ml to use clearly detectable amounts of oxygen. This is equivalent to a turbid culture and is well beyond the levels of cells carried on the rings, which were rinsed repeatedly in PBS before the assay. This, plus the trachea controls and the fact that the trachea rings with the heaviest inocula used the least oxygen, supports the conclusion that the measurement of oxygen uptake results from metabolism of epithelial tissue. The converse situation, whereby tissue catalase could release oxygen from the peroxide liberated by the mycoplasmas, is feasible but highly unlikely since the tissue catalase is intracellular and hence without ready access to substrate, and the amounts of peroxide produced by attached mycoplasmas are extremely small (e.g., 1 µmol/10¹⁵ CFU per h, several orders of magnitude less than are likely to be present).

It appears that oxygen consumption by trachea organ cultures is related to ciliary activity and intactness of the epithelium. Studies designed to examine the kinetics of respiration in mycoplasma-infected tracheas are now in progress. In light of the recent reports (7, 8) of cytopathic activity in membranes of *M. fermentans* and *M. pneumoniae*, it would also be advisable to evaluate such preparations for their effect on tissue respiration. Data presented here and elsewhere (3, 7, 11) indicate that trachea organ cultures may well serve to elucidate the metabolic consequence of infection with *M. pneumoniae*.

ACKNOWLEDGMENTS

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LITERATURE CITED


