Hyperoxia Potentiates *Ureaplasma urealyticum* Pneumonia in Newborn Mice

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Received 7 May 1990/Accepted 6 August 1990

The effect of continuous exposure to 80% oxygen on newborn mice with *Ureaplasma urealyticum* pneumonia was determined. Mice were inoculated intranasally with either *U. urealyticum* or sterile broth and then housed in either 80% oxygen or room air (21% oxygen). The mice were sacrificed at either 7 or 14 days after inoculation. Significantly more mice in the *U. urealyticum* group housed in 80% O₂ than in the room air-exposed group were culture positive 14 days after inoculation (*P* = 0.042), but no difference was found at 7 days. The presence of alveolar macrophages, neutrophils, and lymphocytes and alveolar wall thickness were determined. Overall, the group housed in 80% O₂ and inoculated with *U. urealyticum* had severe pulmonary lesions at both time points, while the lesion severity in the room air-exposed group inoculated with *U. urealyticum* and the group housed in 80% O₂ and inoculated with sterile broth was dependent on the time point. Mortality was significantly higher in the group housed in 80% O₂ and inoculated with *U. urealyticum* than it was in all other groups (*P* < 0.001). Our results indicate that hyperoxia causes the persistence of *U. urealyticum* in the lungs of newborn mice, acutely potentiates the inflammatory response, and turns an otherwise self-limited pneumonia into a lethal disease.

The pathogenesis of bronchopulmonary dysplasia (BPD) has not been completely defined. Although the development of BPD is clearly related to respiratory support, many factors, including pneumonia, may increase the risk of this chronic lung disease (18, 25). We have reported (2) that the isolation of *Ureaplasma urealyticum* within 24 h of birth from the trachea of very-low-birth-weight infants with respiratory disease is associated with an increase in the incidence of BPD (41 versus 82%), death (34 versus 70%), or both. These observations have been confirmed by two other groups of investigators (24, 29).

We have observed that in human infants who require supplemental oxygen and from whom *U. urealyticum* is isolated from their respiratory tracts, this organism is more likely isolated on multiple occasions, and the infants tend to have a worse outcome. We have shown (23) that *U. urealyticum* isolated from the lungs of human neonates produces an acute, self-limited pneumonia in newborn mice. We have also shown (19) that oxidants potentiate respiratory disease because of other mycoplasmas. We speculated that supplemental oxygen increases the pulmonary injury caused by *U. urealyticum* in human neonates and predisposes them to BPD. To investigate a possible interaction between this pulmonary infection and supplemental oxygen, we used the mouse model to test the hypothesis that exposure to 80% oxygen enhances *U. urealyticum* pneumonia in newborn mice. The results indicated that oxygen not only enhances lung lesion severity but also results in organism persistence and death of the mice.

**MATERIALS AND METHODS**

**Experimental design.** Newborn mice were inoculated intranasally with *U. urealyticum* and were reared in either 80% oxygen or room air. A second group of newborn mice was inoculated with sterile broth and was reared under similar conditions. Mice in each group were sacrificed at either 7 or 14 days after inoculation, and their lungs were either quantitatively cultured or processed for histopathological evaluation. The outcomes recorded for this study were persistence of organisms, quantity of organisms recovered, microscopic pulmonary lesions at the 7- and 14-day endpoints, and death.

**Organism.** The isolate of *U. urealyticum* (serotype 10) used in this study was from a tracheal aspirate of an infant with BPD and refractory apnea (23). The organism was isolated in pure culture, passaged twice in 10B medium, and then passed once through pathogen-free newborn C3H/HeN mice. The organism was recovered by a postmortem tracheobronchial lavage, passaged two more times on artificial medium, and suspended in 10B medium at approximately 2 × 10⁶ CFU/ml. One-milliliter portions were frozen at −70°C until use.

**Animals.** Pathogen-free C3H/HeN MTV− pregnant mice were acquired from the National Cancer Institute, Frederick, Md. The mice were shipped behind a bacteriologic filter at 17 to 19 days of gestation. This colony is surveyed on a routine basis for all known murine bacterial, mycoplasmal, and viral pathogens. Each pregnant mouse (dam) was placed into a sterile filter-topped cage with hardwood chip bedding (P. J. Murphy Forest Products, Rochelle Park, N.J.) and provided sterile food (Agway, Inc., Syracuse, N.Y.) and water ad libitum upon arrival at the University of Alabama at Birmingham.

The cages were checked twice daily for the presence of newborn mice (pups). The eight dams that delivered within 24 h of each other and had the greatest number of pups were chosen for the study. Eight standard litters were formed by using a modification of the technique described by Northway et al. (17). Briefly, the pups were separated from the dams and weighed. The six heaviest pups from the first litter were assigned, one by one, to the first six dams. Then, the six heaviest pups from the second litter were assigned in the same manner starting with the seventh dam. In the event that...
one of the litters did not consist of six pups, the heaviest pups from a ninth dam were used to make up for the deficiency. The assignment process continued until six newborn pups were assigned to all eight dams.

After randomization and inoculation, the litters were exposed to the different oxygen tensions in individual cubicles inside sterile Plexiglas cages. Each study group was assigned to a different exposure cage to avoid cross-contamination. Urine and stool dropped through the 0.25-in. (0.6-cm) mesh flooring of each cubicle onto an absorbent pad, which was changed daily. The mice were provided with sterile food and water ad libitum. Sterile aspen shavings (Northeastern Products, Warrensburg, N.Y.) were used for bedding in the exposure cages. Within each inoculum group, the dams were exchanged between the 21 and 80% oxygen tensions every 12 h to prevent oxygen toxicity to the dam and minimize any differences in nursing as a confounding variable.

Twenty-one percent oxygen was provided to the normoxic cages with a hospital-grade compressor (Timetei Instrument Corp., Lancaster, Pa.). Eighty percent oxygen was provided to the hyperoxic cages by blending compressed air with hospital-grade oxygen from a liquid O₂ tank. Both oxygen concentrations were humidified and supplied to each cage at a flow rate of 6 liters/min. The oxygen concentration was analyzed (5577 oxygen analyzer; Hudson, Ventronics Division, Temecula, Calif.) and recorded every 12 h while the study was in progress in the hyperoxic exposure cages and was assumed to be 21% in the room air cages. Humidity, ammonia, and CO₂ were measured daily (gas detector SG-4010; Union Carbide: Linde Division, Post Welding Supply, Birmingham, Ala.) and kept at approximately 50% relative humidity, <15 ppm, and <5%, respectively (1).

These experiments were approved by the Animal Use Subcommittee of the University of Alabama at Birmingham and conformed to the Guide for the Care and Use of Laboratory Animals (20).

**Study group randomization and inoculation.** Four standard litters were randomly chosen by drawing numbers from a box. The pups were inoculated intranasally with 20 μl of *U. urealyticum* inoculum. The pups from the other four standard litters were inoculated intranasally with 20 μl of sterile 10B broth. Two standard litters from each inoculum group were randomly chosen to be reared in 80% oxygen, while the other two standard litters from each group were reared in 21% oxygen. This created four study groups: 80% oxygen exposure and broth inoculation (80B), 80% oxygen exposure and *U. urealyticum* inoculation (80U), 21% oxygen exposure and broth inoculation (21B), and 21% oxygen exposure and *U. urealyticum* inoculation (21U).

**Quantitative cultures and histologic preparation.** The pups were sacrificed with an intraperitoneal injection of pentobarbital. The lungs from one-half of the pups were removed aseptically and disaggregated (Stomacher-80; Dynatech Laboratories Inc., Spartanburg, S.C.) in 2 ml of 10B broth. Three milliliters of 10B broth was added to the resultant slurry and mixed. Serial 10-fold dilutions were made to 10⁻⁵ in 10B broth. Twenty microliters of the slurry and each dilution were plated in duplicate onto A8 agar plates. The plates were incubated at 37°C in 5% CO₂, examined daily, and, if negative, discarded after 7 days.

The lungs from the other half of the pups were removed and inflated with cold 95% ethanol until the acute angles were distended (15). The lungs were then fixed in 95% cold ethanol. After fixation, the lungs were separated into the five individual lobes: right upper, right middle, right lower, azygous, and left. Each lobe was then sectioned along the major bronchus and stained with hematoxylin-eosin.

The lungs of three pups that died prior to the day 7 endpoint were removed and prepared for histology as described above. Two of these three mice were from the 80U group. One pup died 12 h after inoculation, and the other pup died 36 h after inoculation. The third pup was in the 80B group and was killed by the dam 24 h after inoculation. The other pups that died were either partially cannibalized or appeared to have been dead for several hours, and no histological sections were made.

**Grading system.** Previous work has demonstrated that *U. urealyticum* produces pulmonary lesions in newborn mice; these lesions are characterized by both polymorphonuclear and mononuclear cell alveolar infiltrates and thickening of the alveolar walls (23). We have used a crude grading scheme to describe similar lesions seen in murine respiratory mycoplasmosis (16). In this study, representative sections from each study group were examined, and a more descriptive grading scheme was devised based on the grading scheme used in our previous work (16) (Table 1).

The identification mark of each slide was then covered with aluminum foil, and the slides were mixed and graded blindly. Each histologic section was assigned a grade for each of the following characteristics: alveolar macrophages, neutrophils, number and relative size of perivascular and peribronchial lymphocyte patches, and alveolar thickness. Alveolar thickening was not uniform in many cases, so the slides were graded based on the worst focal areas. An overall score for each section was assigned by averaging the individual markers, with the exception of lymphocytes.

**Statistics.** Each experiment was performed five times: twice with a 7-day endpoint (24 pups per group) and three times with a 14-day endpoint (36 pups per group). The culture data were analyzed by the Fisher exact probability test and a t test when appropriate. Analysis of variance techniques with a strip-plot factorial design and least-squares mean technique were used to analyze the histology data. This design took into account the lack of independence of measurements made on the five histological sections from

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**TABLE 1. Scale used for histopathological grading**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Macrophages</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Alveolar wall thickening</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>2-3/alveolus</td>
<td>2-3/alveolus</td>
<td>10-20 cells, one small area moderate in size</td>
<td>Focal, minimal</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1/2 alveoli filled on average</td>
<td>&lt;1/2 alveoli filled on average</td>
<td>One large area (&gt;50 cells) or two or more moderately sized areas</td>
<td>Minimal or focal, moderate</td>
</tr>
<tr>
<td>3</td>
<td>&gt;1/2 alveoli filled on average</td>
<td>&gt;1/2 alveoli filled on average</td>
<td>Two or more large areas</td>
<td>Moderate or focal, marked</td>
</tr>
<tr>
<td>4</td>
<td>All alveoli filled</td>
<td>All alveoli filled</td>
<td>Two or more large areas</td>
<td>Marked</td>
</tr>
</tbody>
</table>

*Each lesion marker was graded on a scale of 0 to 4. The thickness of the alveolar walls was not consistent in some sections, so the alveolar thickness was graded based on the worst focal areas.*

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TABLE 2. Quantitative culture results for 7- and 14-day postinoculation endpoints

<table>
<thead>
<tr>
<th>Group</th>
<th>Sacrifice at day 7</th>
<th>Sacrifice at day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of mice</td>
<td>No. (%) positive</td>
</tr>
<tr>
<td>21U</td>
<td>12</td>
<td>8 (67)</td>
</tr>
<tr>
<td>80U</td>
<td>9</td>
<td>7 (78)</td>
</tr>
<tr>
<td>21B</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>80B</td>
<td>12</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

a CFU, CFU of U. urealyticum per set of lungs.
b NA, Standard deviation not applicable because of only one datum point.
c P = 0.042 by the Fisher exact test.

Thus, the appearance of peribronchial and perivascular lymphocytes was associated with both maturity and inoculation with U. urealyticum. The additional exposure of mice inoculated with U. urealyticum to hypoxia heightened the response.

Significant differences in alveolar wall thickness were found at both the 7-day (P < 0.0001) and 14-day (P < 0.0001) endpoints (Fig. 1 and 2, respectively). The alveolar walls in the lung sections from the 21B group were minimally thickened at 7 days and were considered normal at 14 days (Fig. 1 to 3). Seven days after inoculation, the alveolar wall thickness was significantly greater in all of the groups than in the 21B group, and the alveolar walls in the 80U group were the thickest of all (Fig. 1 and 4). At 14 days postinoculation, the increase in alveolar wall thickness was associated with exposure to hypoxia (Fig. 2).

Significant differences in the overall score (sum) were found at both the 7-day (P < 0.0001) and 14-day (P < 0.0001) endpoints (Fig. 1 and 2, respectively). Overall, the 80U group had significantly more-severe pulmonary lesions at 7 days than the broth inoculated groups did (Fig. 1). Also, both

\[ \text{lesion severity} = \frac{\text{macrophage thickness} + \text{neutrophil thickness} + \text{lymphocyte thickness} + \text{alveolar wall thickness}}{4} \]

FIG. 1. Lung lesion severity in pups sacrificed 7 days after inoculation. The number of pups in each group is represented by n. The bars represent the mean ± standard error. Significant differences (P at least less than 0.05) between groups demonstrated by the least-squares mean technique are represented by the following: significantly different than the 21B group (○), significantly different than the 80B group (●), and significantly different than the 21U group (■).
the 21U and 80B groups had pulmonary lesions more severe than those of the 21B group, as would be expected (Fig. 1). At 14 days postinoculation, the 80U group had significantly more-severe lesions than those of either of the room air-exposed groups (Fig. 2). The 80B group also had more-severe lesions than those of the 21B group at 14 days postinoculation. Thus, while the lesion severity in the 21U and 80B groups depended on the time point, the 80U group continued to have severe pulmonary lesions at both times.

Fig. 5A and B are photomicrographs of the lung section of a pup that died in the 80B group and the lung section of a pup that died 12 h after inoculation in the 80U group, respectively. Both sections demonstrate an alveolar infiltrate of neutrophils and macrophages. Only the section of lung from the pup in the 80U group demonstrated pulmonary hemorrhage and protein exudation. This was also seen in the lung sections of the pup in the 80U group that died 36 h after inoculation. Pulmonary hemorrhage and protein exudation were rarely seen in the sections obtained at the 7- or 14-day time points.

Mortality. Survival in the 80U study group was significantly less than that in the other three groups (P < 0.001 by the log rank test) (Fig. 6). Death was not evenly distributed throughout the study period. It began within 12 h of inoculation, peaked at 3 days, and did not occur in any group after 6 days. Pups became cyanotic and had agonal breathing patterns prior to death, suggesting a pulmonary etiology.

DISCUSSION

U. urealyticum produces a pneumonia in newborn mice which is characterized by a predominantly mononuclear cell infiltrate and alveolar thickening and is similar to the disease in human neonates (23, 28). Our results indicate that exposure of newborn mice inoculated intranasally with U. urealyticum to supplemental oxygen enhances the disease process.

The histology results are likely biased toward less-severe lesions in the 80U group, since the pups that died were likely to have had the most severe lung disease. Lung sections from pups in the 80U group that died prior to the first time...
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FIG. 4. (A) Photomicrograph of lung section of a pup in the 80U group that was sacrificed 7 days after inoculation. The alveolar walls are very thick (arrowhead), and numerous foamy macrophages are present (arrow). Histological grades were as follows: macrophage, 3.0; neutrophil, 0; lymphocytes, 0; alveolar thickness, 4.0. (B) Photomicrograph of a lung section of a pup in the same study group as described above for panel A but sacrificed 14 days after inoculation with U. urealyticum. The alveolar walls are still thickened (arrowhead), and macrophages are present (arrow). Histological grades were as follows: macrophage, 1.0; neutrophil, 0; lymphocytes, 0; alveolar thickness, 1.5. Magnifications, ×100.

point demonstrated severe pulmonary hemorrhage and protein exudation in addition to mononuclear cell and neutrophil infiltrates. These lesions appeared to be severe enough to be the primary cause of death. Thus, pups in the 80U group likely died from their pulmonary lesions and not other causes. In fact, the peak incidence of death in this study occurred coincident with the peak in the pulmonary lesions reported previously (23). It seems reasonable to conclude that the addition of oxygen potentiates the pneumonic process and leads to death in some cases.

FIG. 5. (A) Photomicrograph of a lung section of a pup in the 21U group that was killed by the dam 24 h after inoculation. Neutrophils and macrophages are seen within the alveoli (arrows). (B) Photomicrograph of a lung section of a pup in the 80U group that died 12 h after inoculation. The most impressive feature of this section is the hemorrhage (arrows) and protein exudation in the alveoli.
Death was seen only in the early phase of the pulmonary disease and did not occur after 7 days. This is similar to another mycoplasmal pulmonary infection, murine respiratory mycoplasmosis (16). Murine respiratory mycoplasmosis is a naturally occurring pulmonary infection in rodents caused by Mycoplasma pulmonis. The acute phase in mice is characterized by alveolar infiltrates, hemorrhage, and pulmonary edema. Death occurs frequently during this phase. The chronic phase is characterized by bronchial lesions, and death is not as common. The importance of the chronic phase is that these animals may be more susceptible to other infectious agents and environmental toxins (15, 19). No bronchial lesions were seen in mice inoculated with U. urealyticum.

Several mechanisms may act in concert to cause persistence of U. urealyticum in pups exposed to 80% oxygen and sacrificed 14 days after inoculation. A functional mucociliary system is required for the early phase of pulmonary clearance (11). The discrepancy in organism persistence between the oxygen- and room air-exposed pups cannot be explained by a difference in the early phase of pulmonary clearance, since both oxygen and U. urealyticum decrease mucociliary transport (3, 21). That the number of organisms recovered 7 days after inoculation from the oxygen-exposed pups was not greater than that from the room air-exposed pups also argues that a discrepancy in the early phase of pulmonary clearance does not explain our results. Thus, an alteration in the late phase of pulmonary clearance seems likely.

The late phase of pulmonary clearance involves elimination of microorganisms by phagocytic cells in the lungs, particularly macrophages (11). The cell type that clears U. urealyticum from the respiratory tract is unknown, although these organisms are phagocytosed in vivo by pulmonary macrophages (23). Neutrophils may be disadvantaged if macrophages are the primary cell type that clears U. urealyticum from the respiratory tract. Neonatal rabbit pulmonary macrophages have a decreased rate of bacterial killing which is related to decreased phagocytosis and may be related to a defect in chemotaxis (26). In addition, increased oxygen tensions cause a decrease in the neonatal macrophage respiratory burst, which coincides with the inability to clear Staphylococcus aureus from the lungs (27).

Granulocytes may also be important in the clearance of U. urealyticum from the lungs. Our results demonstrate that a neutrophil infiltrate was present at 7 days in infected mice exposed to room air, while this response was depressed in mice exposed to 80% oxygen. It is not known whether this decreased response was related to the persistence of U. urealyticum in the oxygen-exposed mice. Granulocytopenic mice cannot clear Klebsiella pneumoniae or Pseudomonas aeruginosa effectively, but the clearance of S. aureus is not hampered (22). These data indicate that the phagocytic cell required for pulmonary clearance may depend somewhat on the species of the infecting organism. U. urealyticum has been isolated repeatedly, for up to 189 days, from the respiratory tracts of premature infants with respiratory disease (2). One speculation is that the persistence of U. urealyticum in the respiratory tracts of these premature neonates is related to defects in both neutrophils and alveolar macrophages, and high oxygen tensions may add to these defects (12).

The mechanism of the increased pulmonary injury shown in this study is not known. It is unlikely that this interaction occurs only with U. urealyticum. Whether this interaction is a general response to all microorganisms or is species specific needs to be tested. U. urealyticum produces phospholipases A2 and C, which might affect surfactant function or initiate prostaglandin synthesis, which in turn leads to oxygen radical formation (4). The inflammatory infiltrate produced by U. urealyticum might injure lungs by free radical production by the myeloperoxidase system or elaboration of proteases (7). Oxygen damages type I and type II cells and causes permeability changes and exudation of proteinaceous material into alveoli (3). Control of free radicals or the inflammatory response might decrease the pulmonary injury seen in this model.

Oxygen and photochemical oxidants such as NO₂ and ozone increase mortality in animals exposed to highly virulent aerosolized bacteria (5, 6, 13, 14). Our data demonstrate that U. urealyticum, which has not been shown to kill newborn mice at any of the infectious doses used, can become lethal in the presence of supplemental oxygen.

The experimental design used in this study differs from the typical "infectivity model," which is a standard design used to test the effects of airborne pollutants on the respiratory system (10). The usual experimental protocol is to expose animals to an oxidant for a period of time, often at higher concentrations than those experienced by humans, and then to challenge them with aerosolized bacteria (8, 9). The experimental design used in this study was different, in that the mice were inoculated with U. urealyticum prior to the administration of oxygen, a scenario similar to that in a
human neonate who receives supplemental oxygen for pneumonia or aspiration of infected amniotic or vaginal fluid. The mice were also exposed to an oxygen concentration that was within the range used commonly in human neonatal respiratory care.

These results indicate that oxygen causes persistence of \textit{U. urealyticum} in the lungs of newborn mice, potentiates the inflammatory response, and turns a self-limited pneumonia into a lethal disease. The most severe disease appears to be early in the course of infection, as is the case with other mycoplasma respiratory diseases. The importance of the persistence of \textit{U. urealyticum} in the respiratory tract is only speculative at this point. We suspect, however, that the host may be more susceptible to superinfection, as is the case with other mycoplasma respiratory diseases, and because of the inflammatory response the host may require more respiratory support, further damaging the lungs.

LITERATURE CITED