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Characterization Of Ureaplasma Urealyticum Pneumonia In Human Neonates And A Murine Model.

Dennis Theldon Crouse
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Crouse, Dennis Theldon, Ph.D.

University of Alabama at Birmingham, 1992

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CHARACTERIZATION OF UREAPLASMA UREALYTICUM PNEUMONIA IN HUMAN NEONATES AND A MURINE MODEL

by

DENNIS T. CROUSE

A DISSERTATION

Submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy in the Department of Pathology,
in the Graduate School,
The University of Alabama at Birmingham

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ABSTRACT OF DISSERTATION
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Title Characterization of Ureaplasma urealyticum pneumonia in human neonates and a murine model

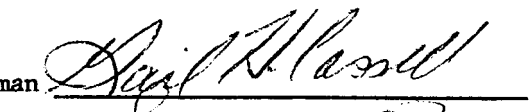
Ureaplasma urealyticum causes pneumonia in newborn infants. We and others have shown that this microbe is associated with chronic lung disease and death in premature infants. Little is known about the disease progression in these infants because the respiratory disease may actually start in utero. The presence of other diseases and confounding therapies also overshadows the disease process. To better understand U. urealyticum disease in premature newborns, we defined chest radiographic changes longitudinally in infected infants. U. urealyticum was associated with radiographic evidence of pneumonia and precocious dysplastic changes (early chronic lung disease (CLD)). Our data support the concept of disease progression from pulmonary infection to chronic lung disease.

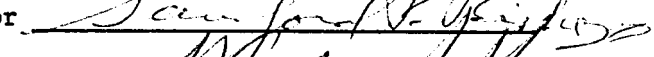
Supplemental oxygen therapy is a characteristic of all infants who develop CLD. To understand the interactions of hyperoxia and U. urealyticum, a mouse model of ureaplasmal pneumonia was used. Infected newborn mice exposed to hyperoxia had increased organism persistence, increased lesion severity, and greater mortality compared to those in room air.

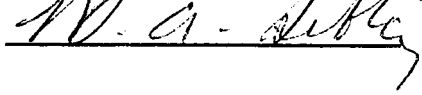
Mature mice are relatively resistant to ureaplasmal pneumonia. We exposed adult and juvenile (10-12 day old) mice to hyperoxia prior to

inoculation with U. urealyticum to determine if hyperoxia would increase their susceptibility to infection. Both adult and juvenile mice were more susceptible to ureaplasmal pneumonia after hyperoxic exposure. The organisms persisted, and pulmonary lesions were worse, but no mortality was seen. Thus, more immunologically mature mice were more susceptible to ureaplasmal pneumonia after hyperoxic exposure.

The susceptibility of ureaplasmas to oxidants was tested since they persisted in a hyperoxic environment. Hydrogen peroxide, hydroxyl radical , and hypohalides are formed by increased cell metabolism and oxygen-dependent microbicidal mechanisms of phagocytic cells. U. urealyticum was subjected to varying concentrations of these oxidants for up to four hours, with and without the catalase inhibitor, aminotriazole. This microbe was very resistant to oxidant injury. Thus, we demonstrated that U. urealyticum is associated with human neonatal pulmonary disease progression, that hyperoxia increases murine susceptibility to this organism, that hyperoxia potentiates the pneumonic process in newborn mice, and that this microbe is resistant to high levels of oxidants normally found in inflammatory loci.

Abstract Approved by: Committee Chairman 

Program Director 

Date 12/22/92 Dean of Graduate School 

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LIST OF ABBREVIATIONS

ATL:	Atelectasis
BHI:	Brain-heart infusion
BPD:	Bronchopulmonary dysplasia
CFU:	Colony forming units
CLD:	Chronic lung disease
EDTA:	Ethylenediaminetetraacetic acid
g:	grams
HMD:	Hyaline membrane disease
H_2O_2:	Hydrogen peroxide
•OH:	Hydroxyl radical
HOI:	Hypohalide form of iodine
HSD:	Honestly significant difference (Tukey's HSD test)
kg:	kilograms
MEM:	Minimal essential medium
M. hominis:	Mycoplasma hominis
M. pulonis:	Mycoplasma pulmonis
ml:	milliliter

MRM:	Murine respiratory mycoplasmosis
MTV:	Mouse tumor virus
NA:	not applicable
NS:	Not statistically significant
PBPD:	Precocious dysplastic changes
PID:	Pelvic inflammatory disease
PIE:	Pulmonary interstitial emphysema
ppm:	Parts per million
PTX:	Pneumothorax
RNICU:	Regional Newborn Intensive Care Unit
SD:	Standard deviation
SE:	Standard error
TTN:	Transient tachypnea of the newborn
UAB:	University of Alabama at Birmingham
ul:	Microliter
U:	Ureaplasma Urealyticum
U. urealyticum:	Ureaplasma Urealyticum
xg:	Times one gravitational force
YE:	Yeast extract

INTRODUCTION

Genital mycoplasmas have been implicated in human disease for over 40 years (115). Showing causality has been problematic because of the ubiquitous nature of these microbes and the fact that they are often isolated with other microorganisms (75,133). Ureaplasma urealyticum is the most common genital mycoplasma isolated from humans (82). This microbe colonizes the lower genital tract of 50 percent or more of women who are of child-bearing age with no apparent ill effects (3,81). It is when this microbe gains access to other areas of the body, especially in immunocompromized hosts, that its disease-producing potential becomes apparent (136).

U. urealyticum has several unique virulence factors that make it a candidate for producing perinatal disease (96). These factors are important not only for their influence on disease production, but also because they provide the basis for the hypotheses tested here. The purpose of the studies reported here was to further define the association of U. urealyticum with chronic lung disease in very premature infants, to elucidate the effects of hyperoxia on the pneumonic process in a mouse model, and to describe the susceptibility of this microorganism to oxidants. What follows is a summary of the literature which provided the background for this work.

History and Growth Characteristics U. urealyticum was first isolated by Maurice Shepard, Ph.D. in 1950 and reported by him four years later (108). These

organisms were isolated from urethral exudates from men with nongonococcal urethritis (NGU). This microbe was first named "T-mycoplasmas" because of the "tiny" colony size on agar plates. This organism gained the name "Ureaplasma urealyticum" in 1974 after it was shown that urea was vital for growth (110). Since then fourteen specific serotypes have been described (94).

Early work with U. urealyticum was hampered by problems with media. Often cultures would not grow or they could not be subcultured (115). It was shown that a pH of 5.5 to 6.5 was the optimal range for growth, unlike the 7.8 to 8.0 pH range for other mycoplasmas (109). Broth medium for ureaplasma growth has changed dramatically over the years. Early broth medium used human ascitic fluid, often with poor results (42). The 10B broth medium in the present studies uses horse serum and is titrated to a pH of approximately 6.0 (112). Agar medium has also gone through many changes (111,113). The A8 agar medium used in the present experiments is supplemented with $CaCl_2$ which reacts with the ammonia produced and makes the ureaplasma colonies appear brown when viewed with transmitted light (114). Both of these media are highly enriched. Although, ureaplasmas grow on, or in, cell-free media, the exact nutrients required for growth are not known completely.

These organisms are approximately the size of a large DNA virus, 0.2 to 0.3 μm in diameter on average, and cannot be seen by traditional light microscopy (9). Approximately 14,000 x g for 40 to 60 minutes is required to concentrate these organisms (146). Unfortunately, broth medium components also sediment at this force. Therefore, traditional methods of washing bacteria

work poorly for these microbes. One to two logs of colony forming units (CFU) of organisms can be lost trying to reduce the media components, often with poor results.

Virulence Factors Ureaplasmas produce or possess several factors which may increase their virulence. These microbes colonize mucosal surfaces, especially those that have ciliated epithelium (19). U. urealyticum can produce ciliostasis in bovine oviductal and fetal tracheal organ cultures, but whether ciliostasis occurs in vivo has not been documented (32,122). U. urealyticum produces a protease which can inactivate IgA, the major immunoglobulin in the conducting airways of the respiratory tract (95). This IgA protease is more specific for the IgA1 isotype (70). This protease also has more specificity for human IgA compared to canine IgA, whereas the protease found in the canine ureaplasmas is more specific for canine IgA (68). Metabolism of urea liberates ammonia, which causes cytopathic effects in tissue culture (123). Ammonia is a respiratory irritant and has been shown to potentiate respiratory disease in rodents (10). Ureaplasmas can inactivate surfactant presumably by the production of phospholipases A and C (31). Activated phospholipases A and C can also liberate arachadonic acid metabolites (103). These metabolites are very important in regulating the pulmonary blood flow by affecting the pulmonary vascular tone (79). Thus, ureaplasmas possess several factors which can alter their local environment and possibly initiate disease or potentiate existing disease.

Ureaplasma Disease in Animals Ureaplasmas have been recovered from cattle, pigs, sheep, goats, dogs, cats, turkeys, chickens, marmosets, and

monkeys (11,17,37,46,47,58,62,66,71,99,100,126,127,131). The genital tract is a common site of isolation in the adult animal, although these organisms can often be isolated from the respiratory tract of newborns (11,100,126,127). Upper respiratory isolation of ureaplasmas from adult animals does occur, but these animals are almost universally asymptomatic (48,71). Thus, proving that this microbe is responsible for disease has often been extremely difficult.

U. diversum is commonly isolated from cattle (6,140). While a large number of animals are colonized and appear to have no adverse effects, this organism is associated with reproductive failure, granular vulvitis, mastitis, placentitis, abortions, and stillbirths, and is one of the etiologic agents for calf pneumonia (34-36,49,90,98,104,). The exact mechanism whereby U. diversum gains access to the upper genital tract is unknown, but most likely it extends directly from the lower genital tract. Intrauterine inoculation of pathogen-free heifers with U. diversum caused infertility, whereas sterile mycoplasma broth did not (72). Inoculation of pathogen-free calves with U. diversum produced a sub-clinical pneumonia characterized histologically by peribronchiolar lymphoid infiltrates (51,61,89). Mononuclear cells are also found in the alveolar regions. The usual clinical feature is failure to gain weight (124). Thus, bovine ureaplasmas are associated with a spectrum of disease; they colonize the urogenital tract of adult animals, cause disease in a selected few, cause reproductive failure, and cause pneumonia in the offspring.

A clinical spectrum of ureaplasmal disease as extensive as that seen in bovine animals has not been described in other animal species. Granular vulvitis, pregnancy loss, and uterine infections associated with ureaplasmas

have been described in sheep (33,77,78). Certain serotypes have been associated with ovine uterine infections and pregnancy loss (77). This raises the possibility that virulence may vary according to serotype.

Extremely important to research as a whole and to the experiments described herein is the endemic mycoplasma pulmonary infection of laboratory rodents, Murine Respiratory Mycoplasmosis (MRM) (15). MRM is a naturally occurring respiratory infection caused by Mycoplasma pulmonis (14). Most commercially bred rodents are infected with this organism, even many of those that are supposed to be pathogen-free (13). The clinical aspects of this disease are slightly different in rats compared to mice. In rats, an acute alveolitis does not occur due to the ability of rats to clear this organism rapidly from the alveolar spaces (14). The disease is characterized by a chronic bronchopneumonia with mononuclear alveolar infiltrates, bronchiectasis,fibrosis, and atelectasis occurring in some strains.

Differences in susceptibility are noted within inbred rat strains. LEW rats are more susceptible to M. pulmonis than are F344 rats (26,27,117,118). LEW rats develop both bronchopneumonia and mononuclear cell infiltrates in the alveoli, while F344 rats develop only bronchopneumonia even when two logs more of CFU of organisms are used to inoculate F344 rats (26). This difference is determined genetically, since environmental factors, age, and the presence of other microorganisms were controlled for.

Mice have both acute and chronic phases of MRM (76). The acute phase is characterized by alveolar inflammation consisting of neutrophils, macrophages, edema, and hemorrhage (76). This phase can be fatal. The

chronic phase is characterized by a bronchopneumonia with peribronchiolar and perivascular lymphoid accumulations. As in rats, inbred mice show differences in susceptibility based on strain. C3H/HeN mice are more susceptible to MRM than C57BL/6 mice because of the decreased mycoplasmal pulmonary clearance (28,87). The clearance of M. pulmonis from C3H/HeN mice has been shown to depend on the particular strain of mycoplasma used (29).

Human Disease U. urealyticum colonizes the lower genital tract of 50 to 80% of women of child-bearing age (73,81). Disease of the lower genital tract is not associated with this colonization (134). Conversely, this microbe colonizes the urethra, and in uncircumcised men, the coronal sulcus, of approximately one-third to one-half of sexually active males, and is associated with NGU in some (137). To prove that U. urealyticum causes NGU, a couple of courageous investigators inoculated themselves, produced NGU, and then eradicated the infection with appropriate antibiotic therapy (132). Isolation rates of U. urealyticum from both sexes are associated with increasing numbers of sexual partners and lower socioeconomic class (55).

The precise role of U. urealyticum in upper genital tract disease in women is elusive. Pelvic inflammatory disease (PID), chorioamnionitis, abruption, infertility, spontaneous abortion, fetal demise, premature birth, and postpartum fever have all been associated with this microbe (41,43,83,85,138). The strongest evidence that U. urealyticum causes upper genital tract disease is found with chorioamnionitis (40,91,116).

Many of the studies that examined the relationship of U. urealyticum and chorioamnionitis are flawed in one or more ways. Many studies found little evidence for disease when chorioamnionitis was compared to lower tract colonization (44,54,69,101). This is not surprising given the high rate of lower genital tract colonization and the fact that chorioamnionitis is caused by several different microorganisms (86). Current data suggest that a subpopulation of women with lower tract colonization develop upper tract disease (16,128). Studies which have taken cultures of the uterine cavity, amniotic fluid, and fetal membranes often fail to take into consideration the duration of labor or the presence, or absence, of intact membranes. The number of microorganisms, mycoplasmas included, that gain access to the upper genital tract significantly rises with prolonged labor or rupture of amniotic membranes and does not necessarily denote a disease process (12,64).

Kundsin et al. studied the association of chorioamnionitis and placental isolation of microorganisms (74). U. urealyticum was significantly associated with chorioamnionitis. Also, U. urealyticum isolation was inversely associated with preterm birth. Hillier et al. documented that isolation of U. urealyticum from the amniotic membranes was significantly associated with chorioamnionitis, even after the duration of labor, duration of membrane rupture, and presence of other bacteria were taken into account (59). Cassell et al. have shown that U. urealyticum can gain access to the intrauterine cavity and cause an inflammatory response in the amniotic fluid as early as 16 to 20 weeks gestation, even in the absence of membrane rupture or clinical amnionitis (16). These organisms persisted for up to two months and the pregnancy resulted in

preterm birth. These associations are very strong, but absolute proof of causality is still awaited.

The importance of genital tract infection in relation to the studies described here, especially chorioamnionitis and placentitis, is the possible adverse effects on the neonate. Maternal infection, especially chorioamnionitis, significantly increases the neonates risk of infection (50). In the study by Cassell et al., where U. urealyticum was isolated from the amniotic fluid at 16 to 20 weeks gestation, one infant was born prematurely, died approximately one day after birth with severe respiratory disease, and had pneumonia documented at autopsy (16). U. urealyticum was isolated from lung tissue obtained postmortem.

U. urealyticum causes pneumonia in human neonates (142). Neonates can be exposed to infectious agents on passage through the birth canal or in utero (8,67,106). U. urealyticum has also been isolated from the tracheas or lungs of infants with pneumonia in the absence of other bacteria, viruses, or chlamydiae (16,92,142). Localization of U. urealyticum in lung lesions has been accomplished by electron microscopy and immunofluorence (92).

Prospective studies have demonstrated an association between U. urealyticum isolation and chronic lung disease. We have shown that in infants with birthweights below 1000 g who have acute respiratory disease, that isolation of U. urealyticum from their trachea within 24 hours of birth is associated with chronic lung disease, death, or both (18). An oxygen requirement for 28 days was used as the definition of chronic lung disease. We cultured these infants for other bacteria, mycoplasmas, viruses, and

trichomonads, and even when these other microorganisms were controlled for, the association of chronic lung disease and U. urealyticum still held. Sanchez and Regan found an association between isolation of U. urealyticum from the throat and chronic lung disease in infants with birthweights of 2 kg or less admitted to the newborn intensive care unit at Columbia University (105). In another study, Wang et al. at McMaster's University found that nasopharyngeal or tracheal isolation of U. urealyticum from infants with birthweights less than 1250 g was significantly associated with chronic lung disease, relative risk 3.4 (145). In a recent study from Israel, Horowitz et al. studied 114 preterm infants and 100 term infants for U. urealyticum isolation (60). Nasopharyngeal colonization was associated with prematurity. None of the healthy term infants was colonized. In 51 preterm infants who were ventilated, 40 percent with U. urealyticum in the tracheal aspirate developed chronic lung disease, whereas only 9.8 percent with negative cultures developed this condition, relative risk 4.1. Chronic lung disease was also significantly associated with nasopharyngeal isolation of U. urealyticum in preterm infants, relative risk 4.0. The strength of the association of respiratory tract isolation of U. urealyticum and chronic lung disease is increased by the fact that this association was found in different centers from different countries with very different patient populations. As with many mycoplasma diseases, causality has not been proven.

Animal Model Rudd et al. used a clinical isolate of U. urealyticum (serotype 10) to develop a newborn mouse model of pneumonia (102). C3H/HeN and C57/BL pathogen-free newborn mice were inoculated intranasally. Pneumonia

peaked at three to five days after inoculation and was then resolved. The disease was not fatal. C3H/HeN mice were more susceptible than the C57/BL mice. The pneumonia was characterized by alveolar accumulations of mononuclear cells with occasional neutrophils. Bronchial lesions were absent. The histologic sections were very characteristic of the lesions seen in lung sections from neonates with U. urealyticum (142). Formation of this model served two purposes: Koch's postulates were fulfilled, and a model to study the interaction of U. urealyticum and host defenses was established.

Susceptible Host U. urealyticum can activate the complement cascade and then be phagocytosed by neutrophils (136). Activation of the phagocytic respiratory burst has been documented by chemiluminescence after phagocytosis (149). Survival of these organisms within activated neutrophils has also been documented (149). Taylor-Robinson et al. have suggested that the phagocytic cell can act as a carrier and transfer U. urealyticum to various parts of the body (136). Thus, neutrophils may actually disseminate ureaplasmal disease in patients who lack the capability to develop specific immunity.

Hypogammaglobulinemic patients are at risk of developing disseminated disease due to ureaplasmas (97). The major sites appear to be joints and urinary tract, although skin, eye, and throat have been recorded (65,120,129,135,147,148). Joint and kidney involvement has been documented in a patient being immunosupressed for a kidney allograft transplant (136). U. urealyticum has also been isolated from the joints of mothers after parturition, possibly because of T-cell suppression (22). Thus, U.

urealyticum can cause invasive disease in patients who are incapable of developing an immunoglobulin response or who have diminished T-cell function.

The maturity of the human neonate's immune system is proportional to their gestational age. The immune system of term infants is more mature than that of the preterm child (20). Certain deficiencies of the neonate's immune system are particularly important in regard to ureaplasmal disease. The ability of the neonate to produce immunoglobulins is diminished compared to the adult (7). This is due in part to several defects. Antigen-presenting cells function poorly in the neonate (57,80). The neonate , especially the preterm infant, has a higher amount of T-cell suppression which down regulates the immune response (56,150). In addition, the production of certain cytokines which up-regulate the immune response may be deficient (139). The neonate relies on transplacentally acquired immunoglobulin to protect against infectious disease (125). The preterm infant born before 32-34 weeks gestation does not receive the complement of immunoglobulins that the term infant receives (63). Thus, preterm infants are deficient in their production and transplacental acquisition of immunoglubulins. Consequently, their ability to mount a specific immune response is greatly diminished. The similarity of neonates to adults with hypogammaglobulinemia is striking, and may explain why neonates are at risk for ureaplasmal disease.

The non-specific defense mechanisms are another aspect of the neonate's immune system which are often overlooked. Components of the complement cascade are at approximately 50 percent of the adult levels in

neonates (2,107). Although phagocytosis is intact in the neonatal neutrophils, chemotaxis and intracellular killing are deficient (141). Certain disease states, such as respiratory disease, make neonates more susceptible to infectious disease (121).

Pulmonary disease is one of the most common maladies in preterm infants (38). Placement of an endotracheal tube causes impairment of the normal pulmonary clearance mechanisms (21,53). Hyperoxia adds to this by causing ciliostasis (30). Evidence of the risk to the infant is the rapidity with which the respiratory tract becomes colonized after endotracheal intubation, usually within 12 to 24 hours (53). Hyperoxia also impairs the intracellular-killing mechanisms of phagocytes (93). Thus, preterm infants who are ill prepared to fight infection are further hampered by their respiratory disease and their therapies.

Summary I have presented evidence to show that U. urealyticum is a human pathogen and is associated very strongly with perinatal disease. Neonates can be exposed to U. urealyticum quite early in gestation with no clinical signs of infection in the mother at the time. Human infants are immunologically immature and those with respiratory disease are further hampered by their disease processes and therapies. People with immune deficits, especially hypogammaglobulinemia, seem to be particularly susceptible to ureaplasmal infection. U. urealyticum can be isolated from infected adults and neonates over a several month period in the absence of appropriate therapy. U. urealyticum is a cause of neonatal pneumonia, which can be fatal and is associated with chronic lung disease in some of those that survive. Finally,

similar spectrums of disease in animals are produced with similar microorganisms. Thus, learning more about the interactions of U. urealyticum and its host and further defining the diseases produced seems prudent. It is with this background and clinical insight that the following studies were devised.

RADIOGRAPHIC CHANGES ASSOCIATED WITH TRACHEAL ISOLATION OF UREAPLASMA UREALYTICUM FROM NEONATES

by

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ABSTRACT

Recent studies show an association between Ureaplasma urealyticum and bronchopulmonary dysplasia (9,24,29). In this study, we examined the pulmonary radiographic findings associated with U. urealyticum. We hypothesized that those infants with birthweights ≤ 1250g and respiratory disease with U. urealyticum present in their tracheal aspirates would have radiographic evidence of more severe pulmonary disease more often than those without this organism present. Two hundred and ninety-two low-birthweight infants who had endotracheal aspirate cultures within 7 days of birth were enrolled into this study. Radiographic outcome variables were pneumonia, early severe bronchopulmonary dysplasia (precocious), and chronic lung disease. Microorganisms were isolated from 128 infants (44 per cent); U. urealyticum was found in 44 (15 per cent). Pneumonia was significantly more common in infants with U. urealyticum than in those without (30 vs 16 per cent, $p = 0.03$). U. urealyticum was also associated with precocious bronchopulmonary dysplasia independent of prematurity, race, and sex (odds ratio 2.2, $p < 0.05$, multiple logistic regression). Tracheal isolation of U. urealyticum within 7 days of birth is associated with pneumonia and precocious bronchopulmonary dysplasia. These data suggest that in infants with U. urealyticum, interventions designed to reduce the severity of lung

disease should be initiated early because of the rapidity with which chronic pulmonary changes take place.

INTRODUCTION

Bronchopulmonary dysplasia is a debilitating pulmonary disease, limited mainly to preterm infants (13). Approximately 1300 new cases annually in the United States were identified in the early 1980's at a projected cost of at least $25 million for care of these infants during their first year (5). The approximate incidence at the end of the decade was 7000 new cases per year with a concomitant increase in costs (18). Even though mortality is now decreasing, up to one-third of these infants die before their first birthday (3). One half of those who survive may have chronic pulmonary dysfunction throughout their life (4). The number of new cases each year is likely to continue to increase as an increased number of very premature infants survive.

Bronchopulmonary dysplasia usually results from the treatment of an acute lung disease (20). Although advances in therapies have been important in the reduction of morbidity and mortality, the key in the treatment of bronchopulmonary dysplasia is prevention. Some diseases or conditions associated with bronchopulmonary dysplasia are hyaline membrane disease, patent ductus arteriosus, and pneumonia (6,8,20,25). Pneumonia is particularly troublesome to the clinician because of the difficulty in diagnosis. We undertook a study to better understand the role of microorganisms in the respiratory tracts of neonates and found that isolation of Ureaplasma urealyticum from the trachea of infants who had birthweights ≤1000 g and who had an oxygen requirement was associated with bronchopulmonary dysplasia

(9). We did not evaluate chest radiographs from the infants in that study. Therefore, the purpose of this study was to correlate findings on chest radiographs, with particular attention to pneumonic and chronic changes, to the culture results of the initial tracheal aspirate obtained within 7 days of birth from low-birthweight infants with respiratory disease.

MATERIALS AND METHODS

Study Design We tested the hypothesis that infants who had Ureaplasma urealyticum isolated from their trachea would have chest radiographic findings consistent with pneumonia, severe chronic lung disease, or both, when compared to infants who did not have U. urealyticum isolated from their trachea. This non-concurrent prospective study was approved by the institutional review board for human use at the University of Alabama at Birmingham.

Study population Infants who had respiratory disease, birthweights ≤ 2500 g, a tracheal aspirate for mycoplasmas, and bacteria within 7 days of birth, who had chest radiographs available for review and who were admitted to the Regional Newborn Intensive Care Unit at the University of Alabama at Birmingham from July, 1985 to February, 1989 were enrolled into this study. February, 1989 was chosen as a stopping point for enrollment because a surfactant trial was beginning.

Specimen Collection The technique for tracheal aspirate collection has been described in detail (9). Briefly, a neonate was intubated with an endotracheal tube, and tracheal secretions were aspirated with a suction catheter inserted through the endotracheal tube. If secretions were scant, 0.5 to 1.0 mls of sterile normal saline was ventilated into the tracheobronchial tree and then the

aspiration was attempted again. The distal portion of the catheter was severed and flushed with 1.0 ml of sterile normal saline. One to two tenths of a ml were placed in 0.9 mls of 10B media and sent to the Diagnostic Mycoplasma Laboratory at UAB and the rest was sent to the UAB hospital clinical microbiology laboratory for routine aerobic bacterial culture. Serial ten-fold dilutions of the aliquot for mycoplasmas was performed on arrival to the laboratory. Twenty microliter samples of each dilution were plated on A8 agar. Cultures were kept for 7 days before being discarded and reported as negative. Routine aerobic bacterial cultures were inoculated into brain-heart infusion broth (BHI) and plated on chocolate, blood, and MacConkey agar. The agar plates were discarded after 48 hours if negative. BHI broth was examined daily for turbidity and gram-stained on day 5 before being discarded as negative. Viral, chlamydial, and anaerobic bacterial cultures were not obtained because of the extremely low yield in this population (9).

Data Collection Demographic and clinical data were obtained from the medical records. Culture data were obtained from the Diagnostic Mycoplasma Laboratory database and the clinical laboratory data reports located in the medical records.

Radiographic Analysis Each infant with respiratory disease had a chest radiograph taken upon admission to the neonatal intensive care unit as part of routine care. Subsequent radiographs were ordered at the discretion of the physicians caring for the infant as clinical status dictated. Clinical interpretation of the chest radiographs were performed daily by a cadre of radiologists on a

daily rotational basis. Thus, as a rule, no radiologist reviewed neonatal radiographs more often than once a week.

For purposes of this study, chest radiographs were retrieved from the archives after the last infant was enrolled into this study. Chest radiographs were reviewed by a pediatric radiologist (G.T.O.) without the benefit of clinical history. The radiographs from approximately 60 infants were interpreted during each session. Radiographs from approximately one per cent of the infants were reinterpreted toward the end of the radiograph reading process, without the knowledge of the radiologist. No discrepancies were found between the first and second interpretations. In addition, approximately 10 per cent of the radiographic interpretations for this study were checked against the original written account of the clinical interpretations. Again, no discrepancies were found.

The primary radiographic outcomes were: Chronic lung disease defined as any grade of bronchopulmonary dysplasia occurring at the end of the fourth postnatal week; precocious dysplastic changes defined as radiographic findings consistent with grade III or IV bronchopulmonary dysplasia, but occurring during the second or early third postnatal week; and pneumonia defined as one or more of the following: (1) radiating peripheral streakiness, (2) coarse patchy parenchymal infiltrates or small diffuse nodules, (3) subtle hazy or nodular basilar infiltrates, or (4) diffuse granularity indistinguishable from hyaline membrane disease, but with better than expected aeration, Figures 1 and 2 (11,12).

Radiographs were also reviewed for the following ad hoc outcome variables: hyaline membrane disease, transient tachypnea of the newborn, pulmonary interstitial emphysema, pneumothorax, and atelectasis (11,27).

Data analysis Infants were subgrouped by the organisms isolated on their initial aspirate. Infants with U. urealyticum isolated from their trachea were grouped together regardless of the other organisms isolated. All infants with organisms other than U. urealyticum isolated were grouped in the bacteria subgroup; this included bacteria and other mycoplasmas. Infants with negative cultures, or negative mycoplasma cultures and missing bacterial cultures (n = 4), were placed in the negative subgroup.

Categorical data were analyzed by Chi-square test for independence. Continuous data were analyzed by analysis of variance technique and intergroup differences were analyzed by Duncan's multiple range test. Multiple logistic regression was used to determine the strength of the association of the culture data and the primary radiographic outcomes, while holding the effects of demographic influences constant. The level of significance was set at $p < 0.05$ for demographic, clinical, and primary outcome variables, and at $p < 0.01$ for other ad hoc outcome variables. All p values less than 0.05 are presented in the tables and figures for completeness.

RESULTS

Study Population A total of 314 infants were eligible for this study. Four infants were excluded because of congenital heart disease or meconium aspiration syndrome. Eighteen of the remaining 310 infants who were eligible for this study did not have radiographs available for review, leaving 292 infants

enrolled into this study. The mean birthweight and gestational age for infants enrolled into this study were 1210 g (S.D. ± 478 g) and 29 weeks (S.D. ± 3.0 wks), respectively. Forty-eight per cent were white and 56 per cent were male. No significant differences in birthweight, gestational age, race, sex, percent Apgar scores below 7 at 1 or 5 minutes, or survival, were detected between infants enrolled and infants without x-rays. Thus, no obvious selection biases were detected between those infants with radiographs and those without.

Culture data The initial tracheal aspirate was obtained at a mean age of 1.3 days (S.D. ± 1.5 days; median 1.0 days, 95 percentile 4 days). No organisms were isolated from 66 per cent of the infants (192/292), but U. urealyticum was isolated from 15 per cent (45/292) and bacteria were isolated from 19 per cent of infants (55/292), (Table 1). U. urealyticum was the most common organism isolated and was in pure culture 71 (32 of 45) per cent of the time. Sixty-seven per cent (37 of 55) of the bacterial isolates were in pure culture. Mycoplasma hominis was the second most commonly isolated organism, next to U. urealyticum, (22/292, 8 per cent). Group B beta hemolytic streptococcus was isolated from eight infants (8/292, 3 per cent), two of which were in combination with U. urealyticum.

Demographic and Clinical Data That infants with U. urealyticum isolated from their trachea were more immature than infants in the bacteria or negative groups is reflected by the significantly lower mean birthweight and gestational age, the higher percentage of infants with 5 minute Apgar scores below 7, and the higher incidence of mechanical ventilation for any reason, (Table 2). These

differences were not significant when the infants were subgrouped by birth-weight.

Primary Radiographic Outcomes Radiographic evidence of pneumonia was twice as common in U. urealyticum positive infants as in U. urealyticum negative infants (30 vs 16 per cent, $p = 0.03$), (Figure 3). To determine if the increased incidence of pneumonia in infants with U. urealyticum could be due to the inclusion of infants who also had bacteria in their trachea, we examined the percentage of infants with and without bacteria in the U. urealyticum group. Infants with bacteria present had a lower incidence of pneumonia than those without (23 vs 32 per cent, respectively).

Pneumonia was over three times as common in the U. urealyticum group as in the bacteria group ($p = 0.01$), and it tended to be more common than in the negative group ($p = 0.08$) when all infants were considered, (Figure 4). Similar relationships were found when infants in the $\leq$ 1250 g birthweight group were examined, (Figure 4). U. urealyticum was associated with pneumonia when the data were analyzed by multiple logistic regression (odds ratio 3.2, $p = 0.05$), but it did not reach statistical significance. Conversely, tracheal isolation of bacteria was not associated with radiographic evidence of pneumonia.

Precocious dysplastic changes were significantly more common in infants with U. urealyticum than in those without ($p = 0.01$) (Figure 3). When all the infants were compared by individual culture groups, precocious dysplastic changes were significantly associated with U. urealyticum compared to negative cultures (63 vs 36 per cent, $p = 0.005$) but not cultures with bacteria isolated (63 vs 41 per cent, $p > 0.05$) (Figure 4). The same associations were

present when only infants with birthweights ≤ 1250 g were analyzed, (Figure 4). Multiple logistic regression was used to control for the influences of prematurity (birthweight), race, and sex, and precocious dysplastic changes were still significantly associated with U. urealyticum ($p<0.5$, odds ratio 2.2). Infants who died before 14 days of age were excluded from this analysis since they did not have a radiograph for review. No significant difference in the incidence of death before 14 days of age was found between culture groups: U. urealyticum 29 percent, bacteria 13 percent, and negative 24 percent ($p = 0.11$). A very small interaction between U. urealyticum and other bacteria was present. When this interaction was accounted for in the logistic regression procedure, the level of significance dropped slightly ($p=0.055$, odds ratio 2.2). As expected, birthweight was inversely associated with early dysplastic changes ($p< 0.0001$). No association was found between sex and early dysplastic changes, while white infants were twice as likely as black infants to have early dysplastic changes when U. urealyticum was isolated from their trachea ($p<0.02$).

Twice as many infants with pneumonia developed precocious dysplastic changes compared to infants without pneumonia (49 per cent 25/51, vs 25 per cent 58/234, $p < 0.001$). The odds ratio for developing precocious dysplastic changes when pneumonia was present was 2.5 ($p = 0.01$, multiple logistic regression). Infants with pneumonia were subgrouped by culture status and the likelihood of developing precocious dysplastic changes was examined. More infants with pneumonia and U. urealyticum developed precocious dysplastic changes (69 per cent, 9/13) than did infants with pneumonia and bacteria (40 percent, 2/5) or infants with pneumonia and negative cultures (42 per cent,

14/33). A similar preponderance for precocious dysplastic changes was found in infants who did not have pneumonia (data not shown). Only trends could be examined because of the small number of infants in each category.

The diagnosis of radiographic chronic lung disease was not associated with any microbe group, although the direction of the interaction between U. urealyticum and bronchopulmonary dysplasia was the same as that with U. urealyticum and precocious dysplastic changes, (Figure 4). Significantly, though, 85 per cent of the infants with precocious dysplastic changes (11/13) developed bronchopulmonary dysplasia, whereas only nine per cent of the infants without these changes (2/20) developed bronchopulmonary dysplasia ($p < 0.0001$).

Ad hoc outcomes: No significant differences at $p < 0.01$ were detected for the ad hoc outcome variables, (Figure 5).

DISCUSSION

Our data show that low birth weight infants with respiratory distress who have U. urealyticum isolated within 7 days of birth from their trachea are likely to have evidence of pneumonia and precocious dysplastic changes on chest radiographs. Precocious dysplastic changes are independent of prematurity, sex, and race. Conversely, bacteria are not associated with these radiographic changes. The association of U. urealyticum and precocious dysplastic changes are confined mainly to the low birthweight group, the group at greatest risk for chronic lung disease.

The label "precocious dysplastic changes" was used to describe findings on radiographs consistent with severe bronchopulmonary dysplasia that occur

at or near the end of the second postnatal week. In reality, this was the progression described in Northway's original paper on bronchopulmonary dysplasia (19). Since that description over 20 years ago, the progression through the stages of bronchopulmonary dysplasia has become slower (5). It is now customary to find that the hazy appearance of stage II disease lasts for several weeks, and that stages III and IV disease occur much later, if at all (5). Thus, we chose "precocious dysplastic changes" to describe the more rapid progression to severe disease typical of the original description of bronchopulmonary dysplasia, but atypical for the disease progression seen today.

The demonstration of an association of U. urealyticum and dysplastic changes in this study is in accordance with previous reports. We described 200 neonates with birthweights ≤ 2500 grams and respiratory disease who had U. urealyticum isolated from their trachea (9). Those with birthweights ≤ 1.0 kg were more likely to develop bronchopulmonary dysplasia, defined by a continuous oxygen requirement until 28 days of age. Sánchez and Regan studied 111 infants at Columbia University with birthweights less than 2.0 kg admitted to a newborn intensive care unit and demonstrated that infants colonized with U. urealyticum had a significantly higher incidence of bronchopulmonary dysplasia: 30 vs 8 per cent (24). They used both abnormal radiographs and oxygen requirement at 30 days of age as the determining factors for bronchopulmonary dysplasia. Wang et al. studied 107 infants at McMaster University with birthweights less than 1250 g and found that colonization with U. urealyticum produced a relative risk of 3.4 for developing

bronchopulmonary dysplasia (29). This effect was found to be independent of prematurity and respiratory support. Bronchopulmonary dysplasia was defined as an abnormal radiograph and abnormal arterial blood gases at 28 days of age, or the radiologist's opinion that this disease would develop based on the last available radiograph of an infant who died before 28 days of age. That these studies performed at three different centers with very different patient populations obtained very similar results attests to the generalizability of these results.

That bacteria were not associated with radiographic evidence of pneumonia is in accordance with previous studies. Sherman et al. demonstrated a correlation of bacteria isolated from the trachea within 8 hours of birth and subsequent bacteremia, but no association was found with radiographic evidence of pneumonia (26). Ablow et al. demonstrated that radiographic findings of group B streptococcal infection were often indistinguishable from the respiratory distress syndrome (1,2).

The most likely explanation why bacteria were not associated with precocious dysplastic changes was that our study was not powerful enough to detect a difference at the $p < 0.05$ level. This assumption is supported by the fact that the direction of the association of tracheal isolation of bacteria and precocious dysplastic changes is in the same direction of that seen with tracheal isolation of U. urealyticum, (Figure 4). A second explanation is that tracheal isolation of bacteria might reflect colonization after birth rather than true infection. Support for this speculation is given in the report by Harris et al who reported that 43 percent of infants with respiratory disease were colonized with

bacteria at the time of intubation (14). Unfortunately, they combined nasopharyngeal and tracheal aspirate results. Additional support is provided by Lau and Hey who reported that bacteria are commonly isolated from the respiratory tract of infants with pulmonary disease and are not specific for pneumonia (17). Differences in the significance of tracheal isolation of U. urealyticum and bacteria might also be explained by the mode of transmission. The major mode of transmission of U. urealyticum is vertically from mother to offspring, whereas bacteria may be transmitted either vertically or nosocomially (22).

Microorganisms were isolated from approximately one-third of the initial tracheal aspirates obtained within 7 days of birth in this study. Two-thirds of the organisms were mycoplasmas. U. urealyticum was significant in that it accounted for 44 percent of the organisms isolated, and 15 percent overall. The microorganisms grouped into the bacteria subgroup were very heterogenous. M. hominis was the most frequently isolated organism, followed by group B streptococcus. The heterogeneity of the bacteria isolated and the frequency with which multiple isolates were obtained suggest that nosocomial colonization probably accounted for many of the positive bacterial cultures.

The results of this study combined with the literature creates a cohesive argument for U. urealyticum being a risk factor, and not just an association, for bronchopulmonary dysplasia. Ureaplasma diversum in calves causes a cuffing pneumonia characterized by alveolar and peribronchiolar mononuclear infiltrates (15,16). We and others have shown that U. urealyticum causes pneumonia in human neonates characterized by both a mononuclear and

polymorphonuclear alveolar infiltrate (7,21,28). Rudd et al. fulfilled Koch's postulates by taking U. urealyticum from an infant with pneumonia and causing pneumonia in a newborn mouse model (23). We have also shown that supplemental oxygen produces increased lesion severity, delayed clearance of the organism, and death in this mouse model of ureaplasma pneumonia (10). Reports by us and others have shown an association between U. urealyticum and bronchopulmonary dysplasia (9,24,29). And pneumonia caused by other microorganisms is associated with development of bronchopulmonary dysplasia (8,25). Finally, the sequence of events in the present study — presence of U. urealyticum in the respiratory tract, presence of radiographic evidence of pneumonia, development of precocious dysplastic changes, and subsequent development bronchopulmonary dysplasia — are associated. Thus, the data are very convincing that clinical trials designed to better define or alter this disease course should be pursued.

Several aspects of this study are important. This study was performed prior to the routine administration of surfactant or steroids and prior to the common administration of erythromycin. Therefore, the disease course described here is unaltered by therapies known to alter the course of neonatal respiratory disease or antibiotics which may eradicate U. urealyticum. Repeating this study would be very difficult for these reasons.

A criticism of this study is that U. urealyticum might promote subsequent bacterial colonization/infection of the respiratory tract in infants who have an increased incidence of precocious dysplastic changes and it is the subsequent bacterial colonization/infection that is associated with precocious dysplastic

changes and not U. urealyticum. This is unlikely because dysplastic lung disease is associated with injury occurring over time. Thus, infants who have bacteria isolated earlier, i.e. those in this study, would have a higher incidence of severe lung disease compared to infants with U. urealyticum. This was not the case. That infants within the U. urealyticum group who also had bacteria isolated did not have a significantly higher incidence of precocious dysplastic changes by logistic regression argues against significant disease caused by bacteria. Even if infants with U. urealyticum did promote bacterial colonization/infection and bacteria were associated with significant disease, it would be U. urealyticum which promotes this process and thus remains a potential target for therapies to alter the disease process.

That routine antibiotic use in the intensive care unit could treat bacterial pneumonia and thus prevent severe pulmonary disease is unlikely. Antibiotics are usually discontinued if the blood cultures are negative, even if a tracheal aspirate is positive. Therefore, most respiratory bacterial isolates were not treated with what would be considered an appropriate course of antibiotics unless sepsis was also present. Even if we assume that antibiotic use prevents severe lung disease caused by susceptible bacteria, the fact that U. urealyticum is associated with severe lung disease and that routine antibiotic usage is ineffective against this organism still exists. In fact, this assumption argues for the identification of microorganisms that might be associated with severe lung disease and which are not routinely treated with common antibiotics because appropriate therapy might prevent severe lung disease. Thus, we think that the

inability to associate bacteria with severe lung disease may actually strengthen our argument for the association of U. urealyticum and severe lung disease.

Another criticism of this study is that the radiographs were not obtained at standardized times. We think that this is a very minor problem because the disease processes associated with our primary outcome variables are quite severe, and it is standard therapy to follow these disease processes with frequent radiographs. Also these disease processes are a continuum and radiographs taken even 3 to 4 days apart are extremely unlikely to miss one of the primary outcomes.

In summary, these data show that U. urealyticum isolated from the trachea of premature neonates with respiratory disease within 7 days of birth identifies a group of infants who are likely to have radiographic evidence of pneumonia and who will progress to have severe lung disease by two weeks of birth, as evidenced by radiography. This finding is important since it demonstrates how rapid severe pulmonary changes take place in these infants when U. urealyticum is also isolated from their trachea. These data also suggest that any therapy to reduce the risk or severity of bronchopulmonary dysplasia should be instituted as soon as possible because of the rapidity with which severe pulmonary disease develops in these infants. Therapeutic trials with the elimination of bronchopulmonary dysplasia as the major outcome may be unrealistic, especially if small sample sizes are used, due to the rapidity with which these changes take place. A more realistic goal might be a reduction in the severity of chronic lung disease and/or reduction in death.

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TABLE 1

Culture Results

Microorganism	Incidence (per cent)
None	192 (66)
Any Microorganism	100 (34)
Ureaplasma urealyticum [1]	45 (15)
Other Bacteria [2]	55 (19)
Mycoplasma hominis	22 (8)
Group B streptococcus	6 (2)
Staphylococcus epidermidis	7 (2)
Coagulase negative staphylococci	9 (3)
Streptococcus viridans	3 (1)
Escherichia coli	1 (1)
Enterococcus	2 (1)
Propionibacterium acnes	4 (1)
Other [3]	8 (3)

1. Includes 13 infants with bacteria isolated concurrently: group B streptococcus-2, Mycoplasma hominis-2, Staphylococcus epidermidis-2, Streptococcus viridans-2, and coagulase negative staphylococcus-2, and one each of Candida albicans, Propionibacterium acnes, Staphylococcus aureus., and Peptostreptococcus sp.

2. Total for individual microorganisms and "other bacteria" total will not agree because of multiple isolates in some cultures.

3. Includes one each of Bacillus species, Bacteroides species, Candida albicans, Diphtheroid species, Proteus mirabilus, Pseudomonas species, Staphylococcus Aureus, and Peptostreptococcus species.

TABLE 2

Demographic and Clinical Data[1]

	Total n=292				≤1250 g n=177			
	NEGATIVE	UREAPLASMA	BACTERIA	p[2]	NEGATIVE	UREAPLASMA	BACTERIA	p[2]
Number (percent)	192(66)	45(15)	55(19)		120(68)	34(19)	23(13)	
Birthweight, grams	1215	1062[a]	1314[a]	.05	900	860	840	NS
(range)	(505-2490)	(510-2400)	(604-2410)		(505-1250)	(510-1240)	(604-1250)	
Gestational Age, weeks	29	28[a]	30[a]	.05	27	27	27	NS
(range)	(22-38)	(23-36)	(22-38)		(22-32)	(23-31	(23-31)	
Sex male	55	62	55	NS	52	68	52	NS
Race white	52	44	36	NS	47	44	39	NS
Apgar 1 min (< 7)	63	78	63	NS	76	79	96	NS
Apgar 5 min (< 7)	30[b]	53[b]	24[b]	.01	39	56	52	NS
Survived Hospital	76	71	87	NS	69	65	74	NS
0_2 for 28 days	21	29	22	NS	27	38	48	NS
Ventilation	75	87	65	NS	87	91	91	NS
Apnea	56	60	44	NS	67	71	65	NS

1 Variables in table represent percent unless otherwise noted.

2 Probability of difference within groups.

a. Significantly different from ureaplasma group at $p<0.05$.

b. Significantly different from ureaplasma group at $p<0.01$.

Figure 1. Chest radiograph of a two day old infant showing many of the features consistent with pneumonia. The lung fields are hazy with some streaky densities. The diaphragmatic excursion is good, signifying good compliance, as opposed to small lung fields seen with diseases such as hyaline membrane disease which have poorer compliance.

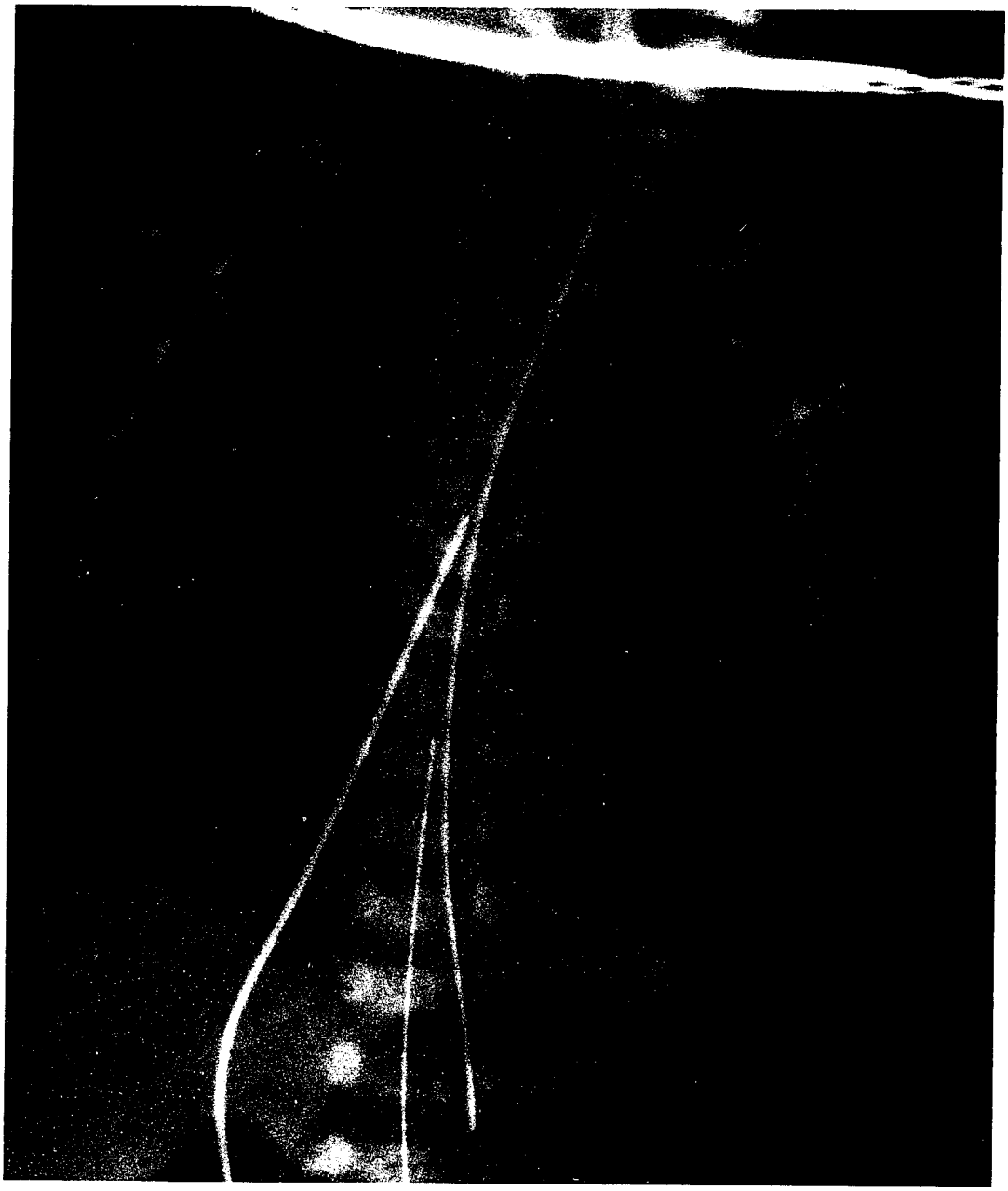


Figure 2. Chest radiograph of a 15 day old infant with precocious dysplasia changes. The lung fields have differential areas of aeration. Streaky areas of atelectasis, or early scarring, are noted along with areas of cystic, emphysematous areas. The lungs appear overaerated, which correlates clinically with air trapping. This radiograph is consistent with grade III bronchopulmonary dysplasia, but is occurring at a much earlier time than usual.

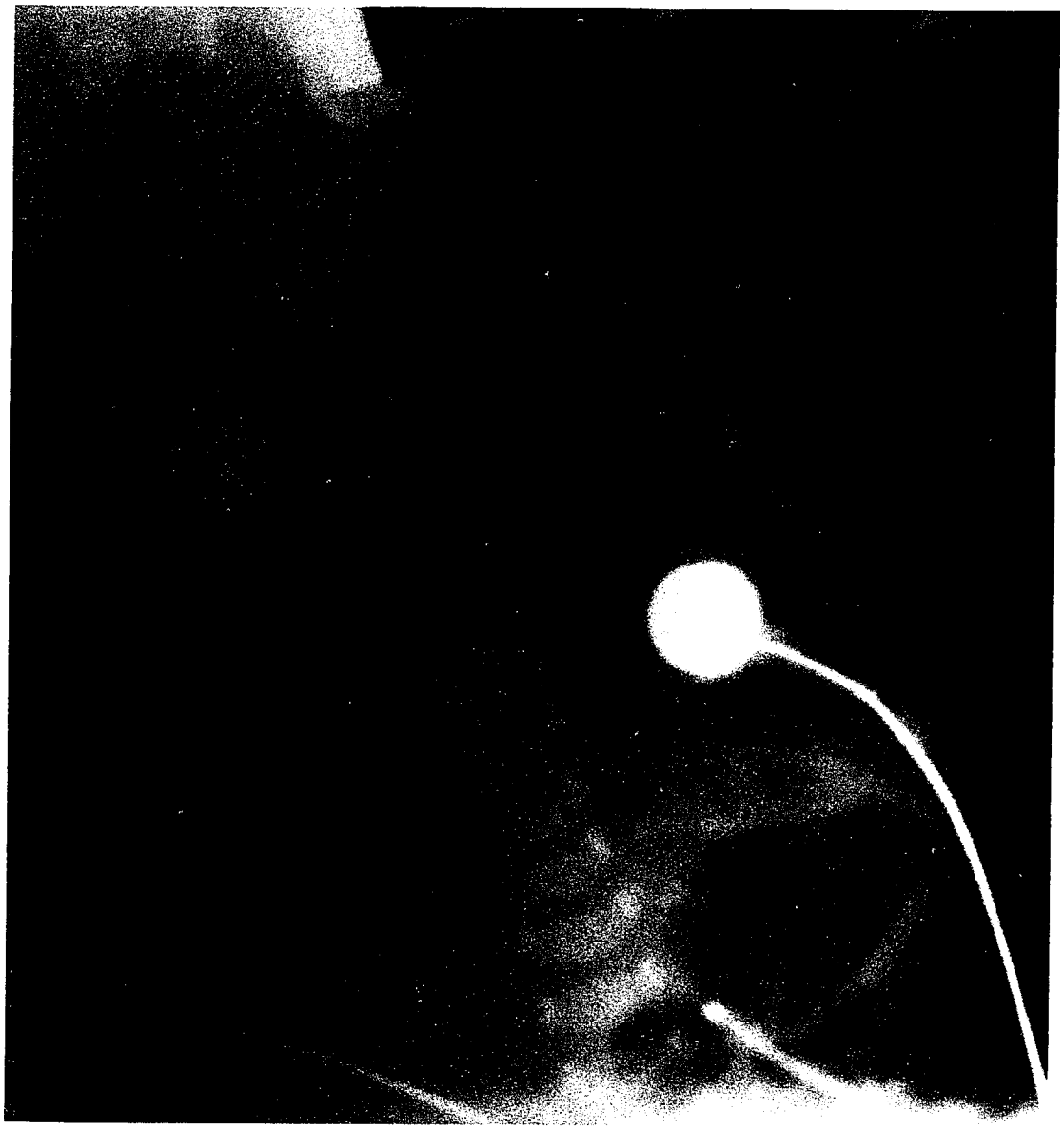


Figure 3. Percentage of infants with primary radiographic outcomes grouped according to whether U. urealyticum was present or absent. Pneu - radiographic evidence of pneumonia, PBPD - precocious dysplastic changes, CLD - chronic lung disease. All infants are depicted in A, and those infants with birthweights ≤1250 grams are depicted in B. a = $p < 0.05$ and b = $p < 0.01$. Numbers in each bar represent number of infants in denominator.

Figure 3.

PRIMARY RADIOGRAPHIC OUTCOMES FOR INFANTS WITH AND WITHOUT U. UREALYTICUM

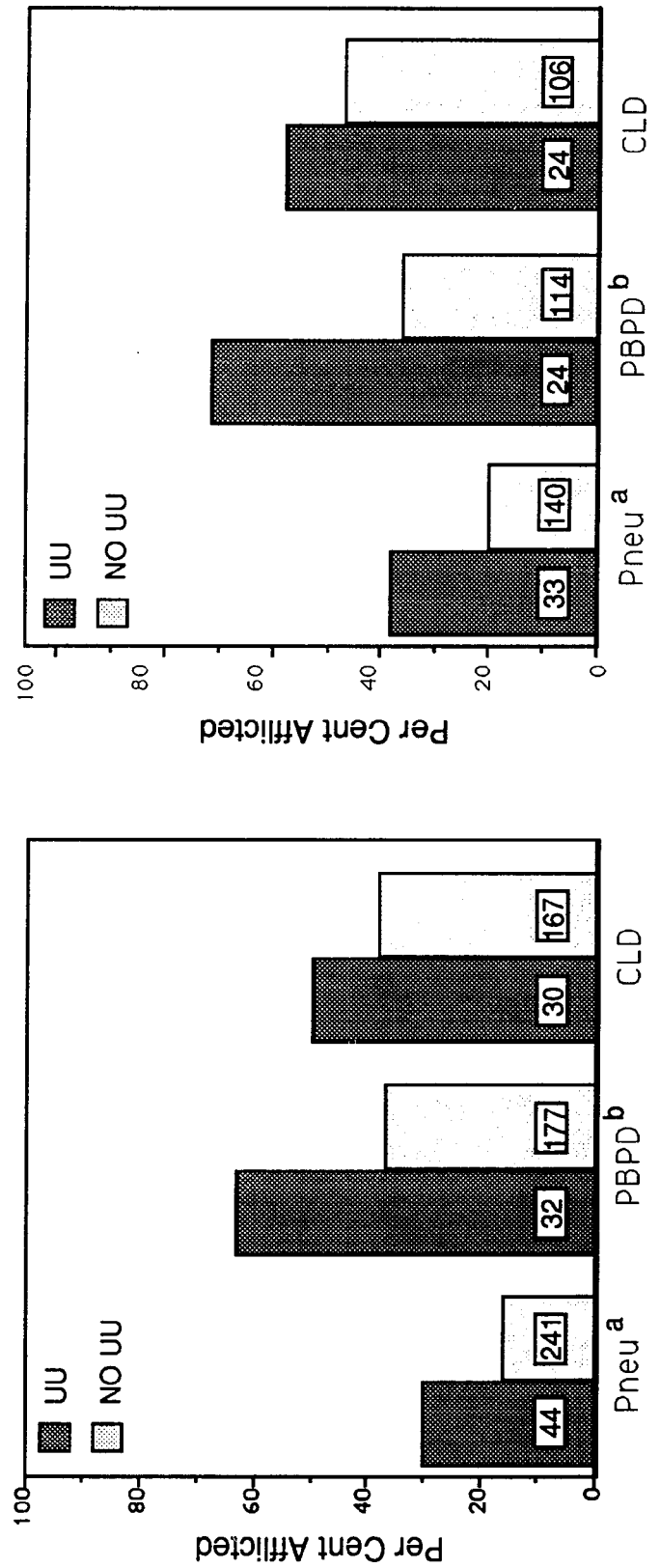
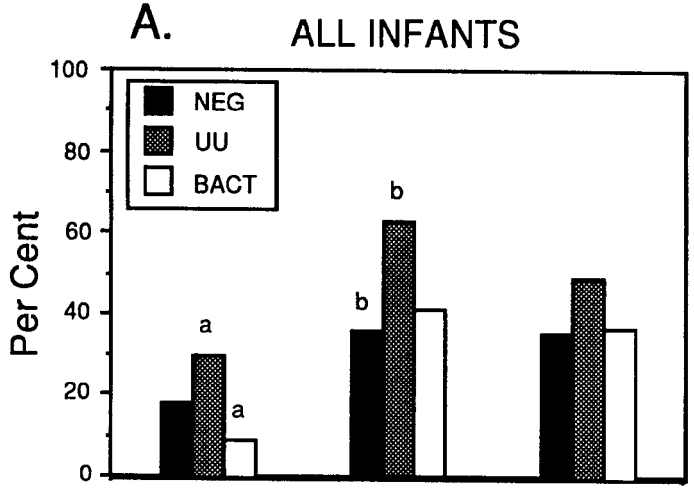


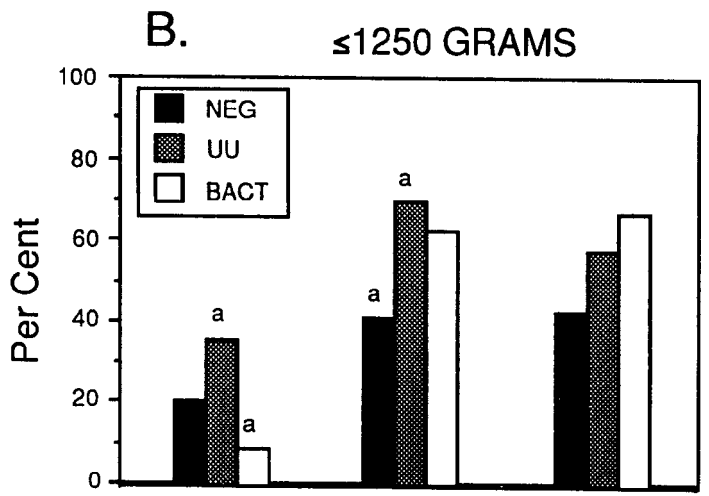
Figure 4. Percentage of infants with primary radiographic outcomes by culture groups. The number of infants in each culture group varied between outcome groups because of death, therefore, the raw data is presented below each graph. All infants are depicted in A, and those infants with birthweights ≤1250 grams are depicted in B. a=$P<0.01$, b=$P<0.05$ by chi-square. All other comparisons were not statistically significant at $P<0.05$.

Figure 4

PRIMARY RADIOGRAPHIC OUTCOMES



NEG	33/187	50/140	48/132
UU	13/44	20/32	15/30
BACT	5/54	15/37	15/35

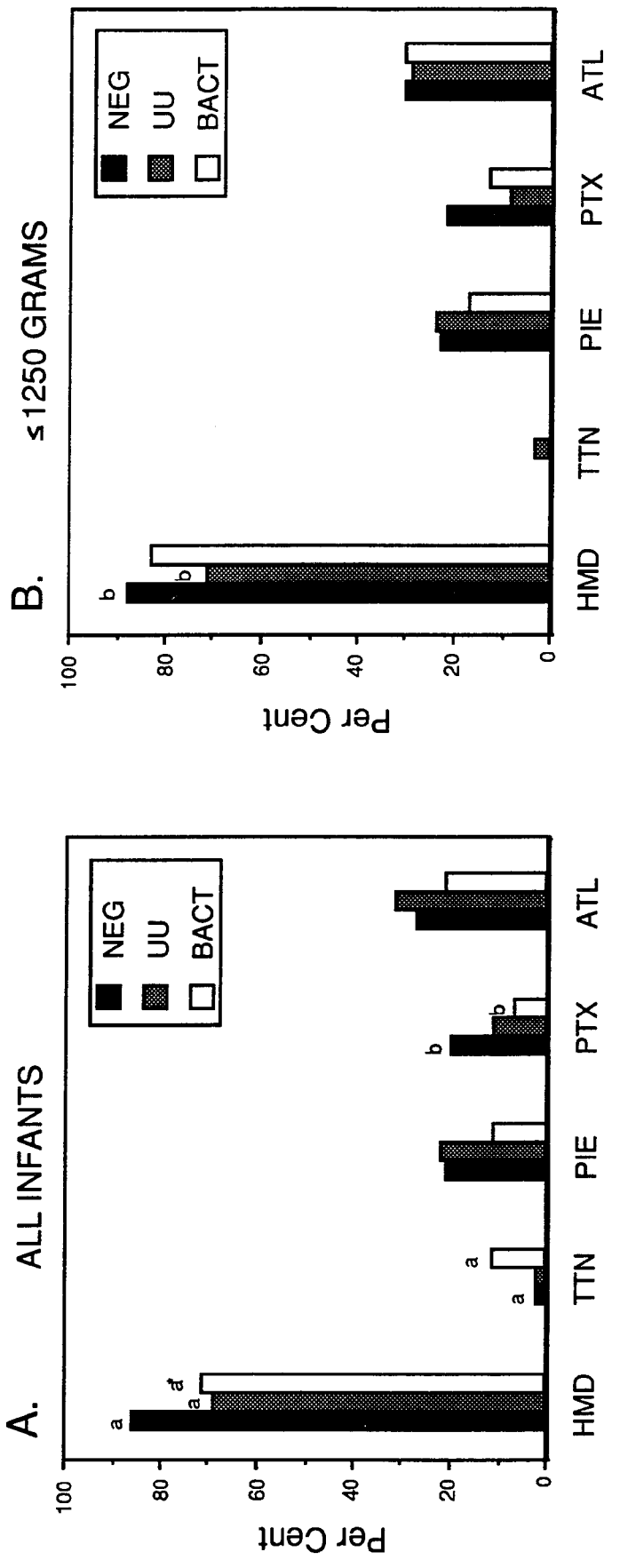


NEG	25/117	39/95	38/88
UU	12/33	17/24	14/24
BACT	2/23	12/19	12/18

Figure 5. Percentage of infants with ad hoc radiographic outcomes by culture group. The standard abbreviations are: HMD - Hyaline membrane disease, TTN - Transient tachypnea of the newborn, PIE - Pulmonary interstitial emphysema, PTX - Pneumothorax, and ATL - Atelectasis. All infants are depicted in A, and those infants with birthweights ≤1250 grams are depicted in B. The number of infants in each culture group varied little between outcome groups, data not shown (data available). a=$P<0.01$ and b=$P<0.05$ by chi-square. All other comparisons were not statistically significant at $P<0.05$.

Figure 5

AD HOC RADIOGRAPHIC OUTCOMES



HYPEROXIA POTENTIATES UREAPLASMA UREALYTICUM PNEUMONIA IN NEWBORN MICE

by

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ABSTRACT

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% O_2 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, lymphocytes, and alveolar wall thickness was determined. Overall, the group housed ib 80% O_2 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% O_2 and inoculated with sterile broth was dependent on the time point. Mortality was significantly higher in the group housed in 80% O_2 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.

INTRODUCTION

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 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 (2). 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 that U. urealyticum isolated from the lungs of human neonates produces an acute, self-limited pneumonia in newborn mice (23). We have also shown that oxidants potentiate respiratory disease caused by other mycoplasmas (19). 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 indicate that oxygen not only enhances lung lesion severity, but also results in organism persistence, and death of the mice.

MATERIALS AND METHODS

Experimental Design. One group of newborn mice was 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 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 resuspended in 10B medium at approximately 2×10^8 CFU/ml. One-milliliter aliquots were frozen at -70°C until use.

Animals. Pathogen-free C3H/HeN MTV- pregnant mice were acquired from the National Cancer Institute, Frederick, Maryland. The mice were shipped behind a bacteriologic filter at 17 to 19 days 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 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 to minimize any differences in nursing as a confounding variable.

Twenty-one percent oxygen was provided to the normoxic cages by a hospital grade compressor (Timeter Instrument Corp., Lancaster, PA.). Eighty percent oxygen was provided to the hyperoxic cages by blending compressed air with hospital-grade oxygen from a liquid O_2 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 CO_2 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 UAB Animal Use Subcommittee and conform 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., Spartenburg, S.C.) in 2 ml of 10B broth. Three milliliters of 10B broth were added to the resultant slurry and mixed. Serial ten-fold dilutions were made to 10^{-5} in 10B broth. Twenty microliters of the slurry and each dilution were plated in duplicate on A8 agar plates. The plates were incubated at 37°C in 5% CO_2, 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 mice were from the 80U group. One 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 canabalized or appeared to have been dead for several hours, so histological sections were not 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 with the Fisher exact probability test and a t-test where appropiate. Analysis of variance techniques with a strip-plot factorial design and least squares means 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 each pup. The level of significance was set at $p \leq 0.05$ and the F-statistic was used to test the model.

The mortality data were analyzed with a Kaplan-Meir survival plot and log rank analysis. Twelve pups in the 21B group, two standard litters, were excluded from the mortality analysis because of maternal cannibalism.

RESULTS

Cultures. U. urealyticum was not isolated from any of the pups inoculated with sterile broth (Table 2). U. urealyticum was isolated from approximately the same number of pups of groups 80U and 21U, regardless of the oxygen environment, at 7 days (7 of 9 pups in the 80U group versus 8 of 12 pups in the 21U group, no significant difference), while at 14 days, significantly more pups were culture positive in the hyperoxic group (6 of 17 versus 1 of 17 pups; $p = 0.042$, Fisher exact test). The number of organisms recovered from infected pups was not significantly different at either time point. This lack of significance did not change if the denominator was all pups inoculated, instead of just the pups with positive cultures. For both the 80U and 21U groups, there was a decline in the number of organisms present in the lungs at 14 days compared with that at 7 days.

Histology. Significant differences in macrophage concentration were found at both 7-day ($p < 0.0001$) and 14-day ($p < 0.0001$) day time points (Fig. 1 and 2, respectively). At both time points, the presence of alveolar macrophages in the lung sections of mice was significantly associated with exposure to hyperoxia, while it was relatively rare in the 21B group (Fig. 1 to 4). An increased number of alveolar macrophages were also demonstrated in lung sections from mice in the 21U group 7 days after inoculation but not 14 days after inoculation (Fig. 1 and 2, respectively).

Significant differences in neutrophil concenrations were found only at the 7-day time point ($p < 0.0001$) (Fig. 1). Alveolar neutrophils were extremely rare in lung sections from mice in the 21B group (Fig. 1 to 3). They were also rare in lung sections from mice in the hyperoxic groups (Fig. 1, 2, and 4). Seven days after inoculation, there were significant numbers of neutrophils present in the lungs of mice in the 21U group, but this was not so 14 days after inoculation (Fig. 1 and 2, respectively).

Significant differences in lymphocyte concentration were found at both the 7-day ($p < 0.5$) and 14-day ($p < 0.0001$) endpoints (Fig. 1 and 2, respectively). The appearance of peribronchial and perivascular lymphocytes was associated with age (Fig. 1 and 2). At both times, significantly more lymphocytes were present in the 80U group than in the broth-inoculated groups (Fig. 1 and 2). At 14 days postinoculation, significantly more lymphocytes were present in the 21U group than the broth-inoculated groups (Fig. 2). 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 hyperoxia heightened the response.

Significant differences in alveolar wall thickness were found at both the 7-day ($p < 0.0001$) and 14-day ($p < 0.001$) endpoints (Fig. 1 and 2, respectively). The alveolar walls in the lung sections from the 21B group were minimally thickened at 7 days and 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 hyperoxia (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 the 21U and 80B groups had pulmonary lesions more severe than 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 from 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 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 predominately 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 point demonstrated severe pulmonary hemorrhage and protein exudation in addition to mononuclear cell and neutrophil infiltrates. These lesions appeared severe enough to be the primary cause of death. Thus, pups in the 80U group likely died from their pulmonary lesions and not from 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.

Death was only seen 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). Neonates 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 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 unknown whether this decreased response is 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 persistance of U. urealyticum in the respiratory tract 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 NO_2 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 that experienced by humans, and then 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 of 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 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 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. Because of the inflammatory response the human host may require more respiratory support, further damaging the lungs.

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TABLE 1

HISTOPATHOLOGICAL GRADING SCALE

GRADE	0	1	2	3	4
MACROPHAGES	NONE	2-3 /CELLS ALVEOLI	< 1/2 ALVEOLI FILLED ON AVGERAGE	> 1/2 ALVEOLI FILLED ON AVGERAGE	ALL ALVEOLI FILLED
NEUTROPHILS	NONE	2-3 /CELLS ALVEOLI	< 1/2 ALVEOLI FILLED ON AVGERAGE	> 1/2 ALVEOLI FILLED ON AVGERAGE	ALL ALVEOLI FILLED
LYMPHOCYTES	NONE	10-20 CELLS ONE SMALL AREA	MULTIPLE SMALL AREAS OR 1 AREA MODERATE IN SIZE	1 LARGE AREA (>50 CELLS) OR 2 OR MORE MODERATE SIZED AREAS	2 OR MORE LARGE AREAS
ALVEOLAR WALL THICKENING	NONE	FOCAL, MINIMAL	MINIMAL OR FOCAL MODERATE	MODERATE OR FOCAL MARKED	MARKED

TABLE 2. QUANTITATIVE CULTURE RESULTS

	7 DAY SACRIFICE			14 DAY SACRIFICE		
GROUP	n	# POS (%)	AVG. CFU (+/-SD)	n	# POS (%)	AVG. CFU (+/-SD)
21U	12	8 (67)	3.76×10^7 (6.44×10^7)	17	1 (6)	1.25×10^2 (NA*)
80U	9	7 (78)	3.02×10^6 (4.37×10^6)	17	6 (35)●	3.33×10^2 (1.51×10^2)
21B	6	0 (0)	0	18	0	0
80B	12	0 (0)	0	18	0	0

Table 2. Quantitative culture results for 7 and 14 day post-inoculation endpoints.

CFU - colony forming units of U. urealyticum per set of lungs

SD - Standard deviation

NA - Not applicable because of only one data point

● p = 0.042 Fisher's Exact Test

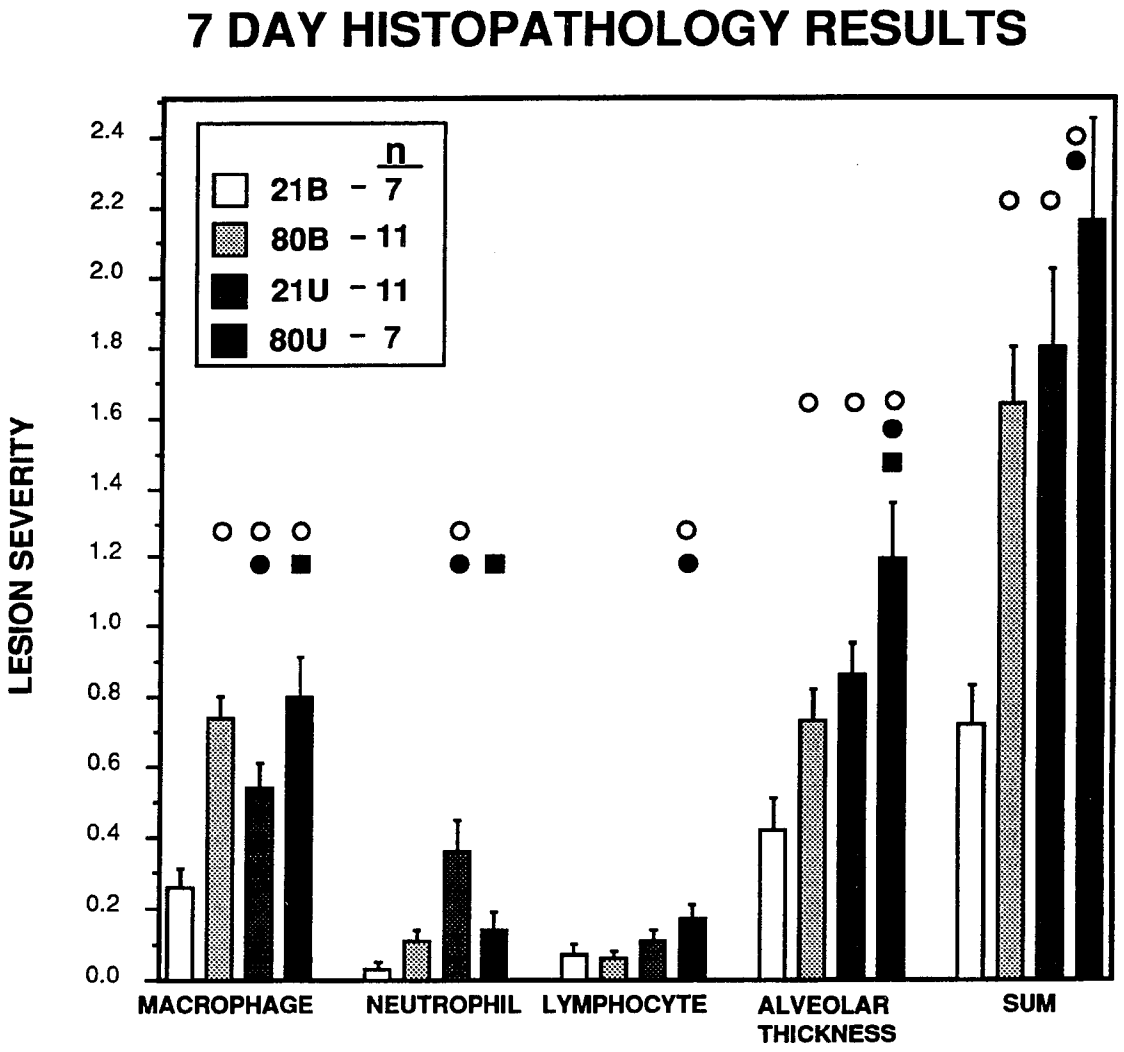


FIG. 1. Graph of lung lesion severity from pups sacrificed 7 days after inoculation. The number of pups in each group is represented by n. The bars represent the mean plus the S.E. Significant differences (p at least < 0.05) between groups demonstrated by the least squares mean technique are represented by the following:

O - Significantly different than 21B group

● - Significantly different than 80B group

■ - Significantly different than 21U group

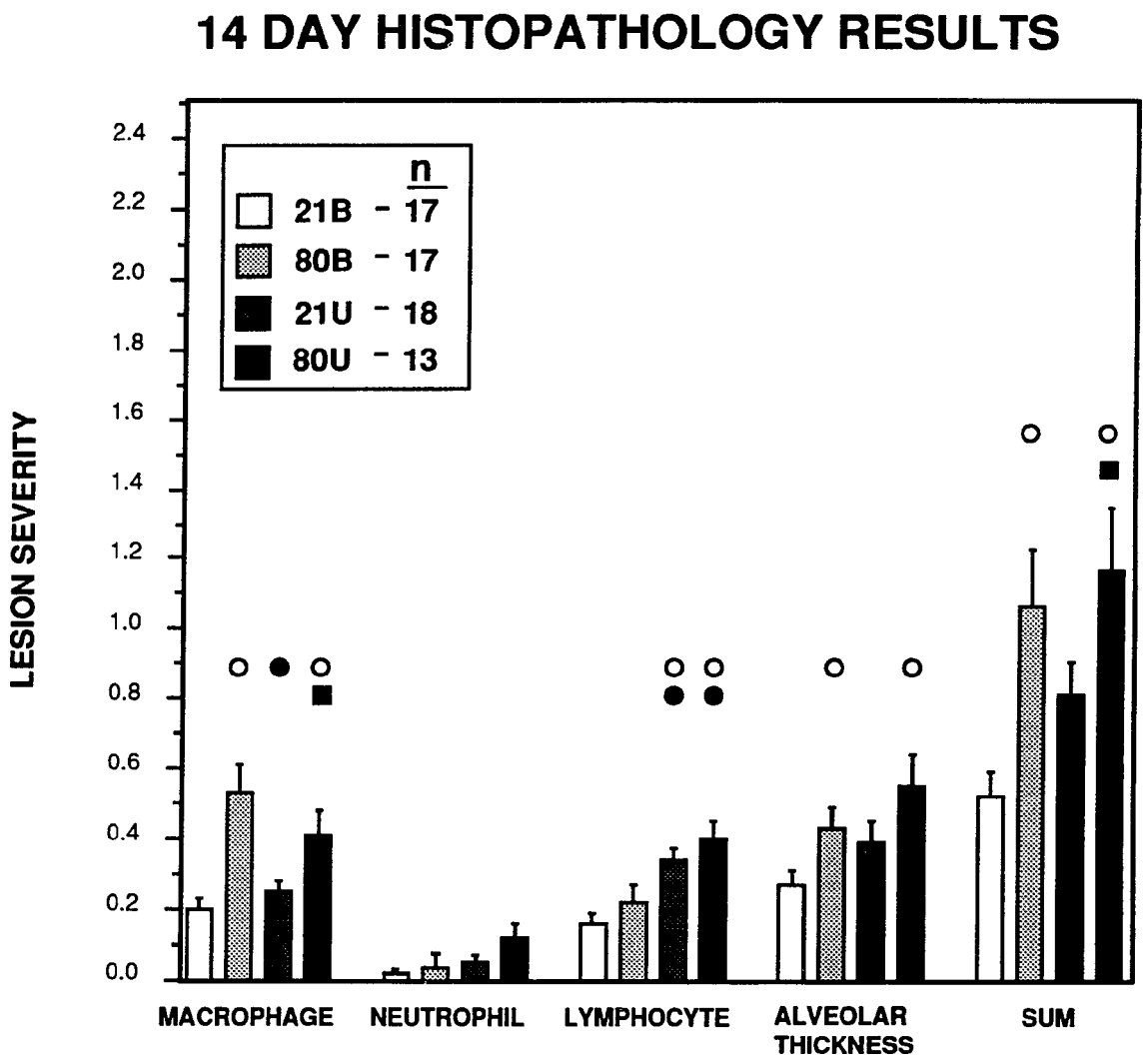


FIGURE 2. Graph of lung lesion severity from pups sacrificed 14 days after inoculation. The number of pups in each group is represented by n. The bars represent the mean plus the S.E. Significant differences (p at least < 0.05) between groups demonstrated by the least squares mean technique are represented by the following:

O - Significantly different than 21B group
● - Significantly different than 80B group
■ - Significantly different than 21U group

FIGURE 3. A. Photomicrograph of lung section of room air broth-inoculated pup sacrificed 7 days after inoculation. The alveolar walls are slightly thickened (small arrow) and few alveolar macrophages are present (large arrow). Histological grade: macrophage - 0.5, neutrophil - 0, lymphocytes - 0, and alveolar thickness - 1.0. B. Photomicrograph of lung section of pup from the same study group sacrificed 14 days after inoculation with broth. The alveolar walls are uniformly thin and no alveolar macrophages are present. Histological grade: all markers - 0. (Magnification 100 x)

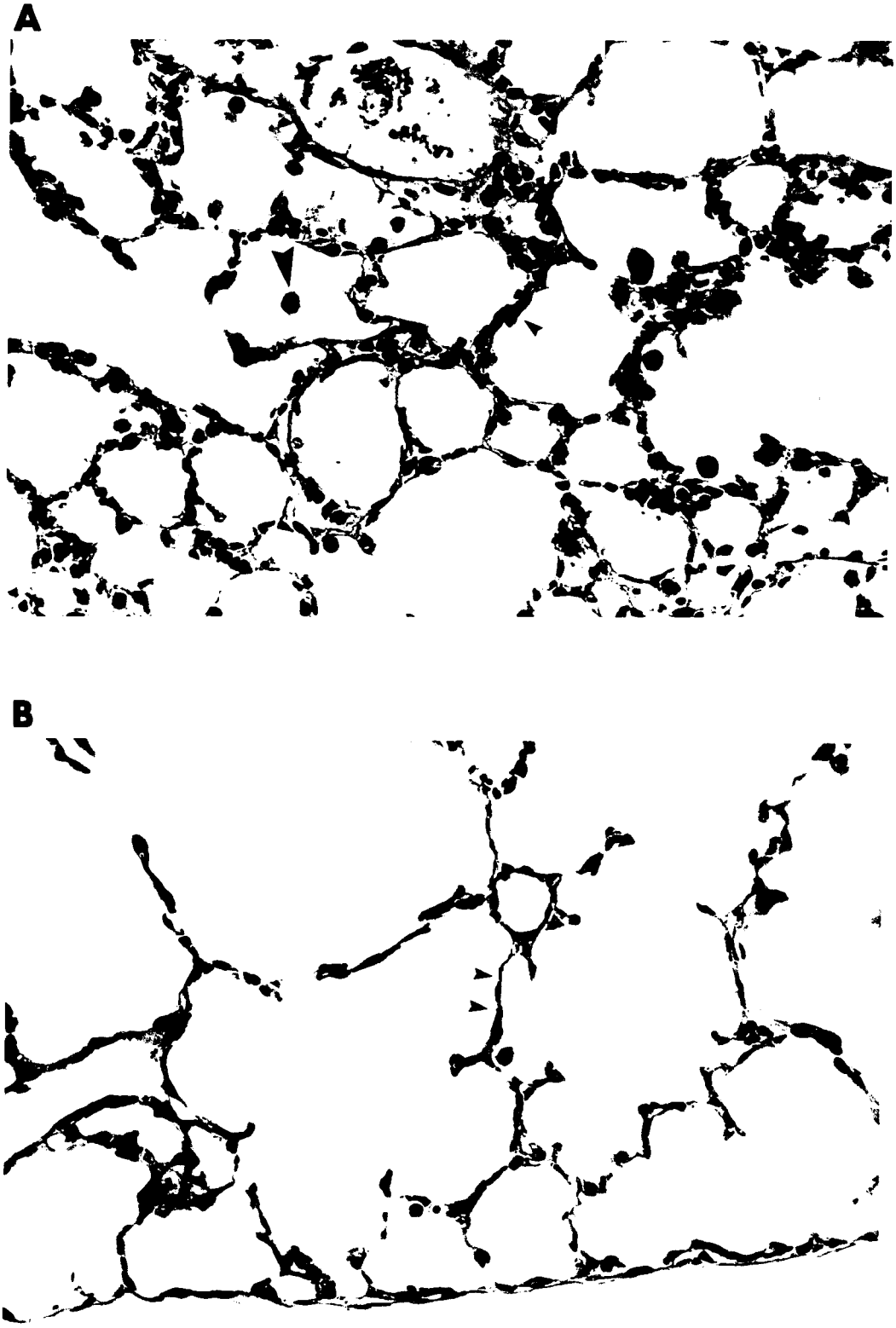

A
B

FIGURE 4. A. Photomicrograph of lung section of oxygen-exposed ureaplasma-inoculated pup sacrificed 7 days after inoculation. The alveolar walls are very thick (small arrow) and numerous foamy macrophages are present (large arrow). Histological grade: macrophage - 3.0, neutrophil - 0, lymphocytes - 0, and alveolar thickness - 4.0. B. Photomicrograph of lung section of a pup in the same study group sacrificed 14 days after inoculation with U. urealyticum. The alveolar walls are still thickened (small arrow) and macrophages are present (large arrow). Histological grade: macrohage - 1.0, neutrophil - 0, lymphocytes - 0, and alveolar thickness - 1.5. (Magnification 100 x)

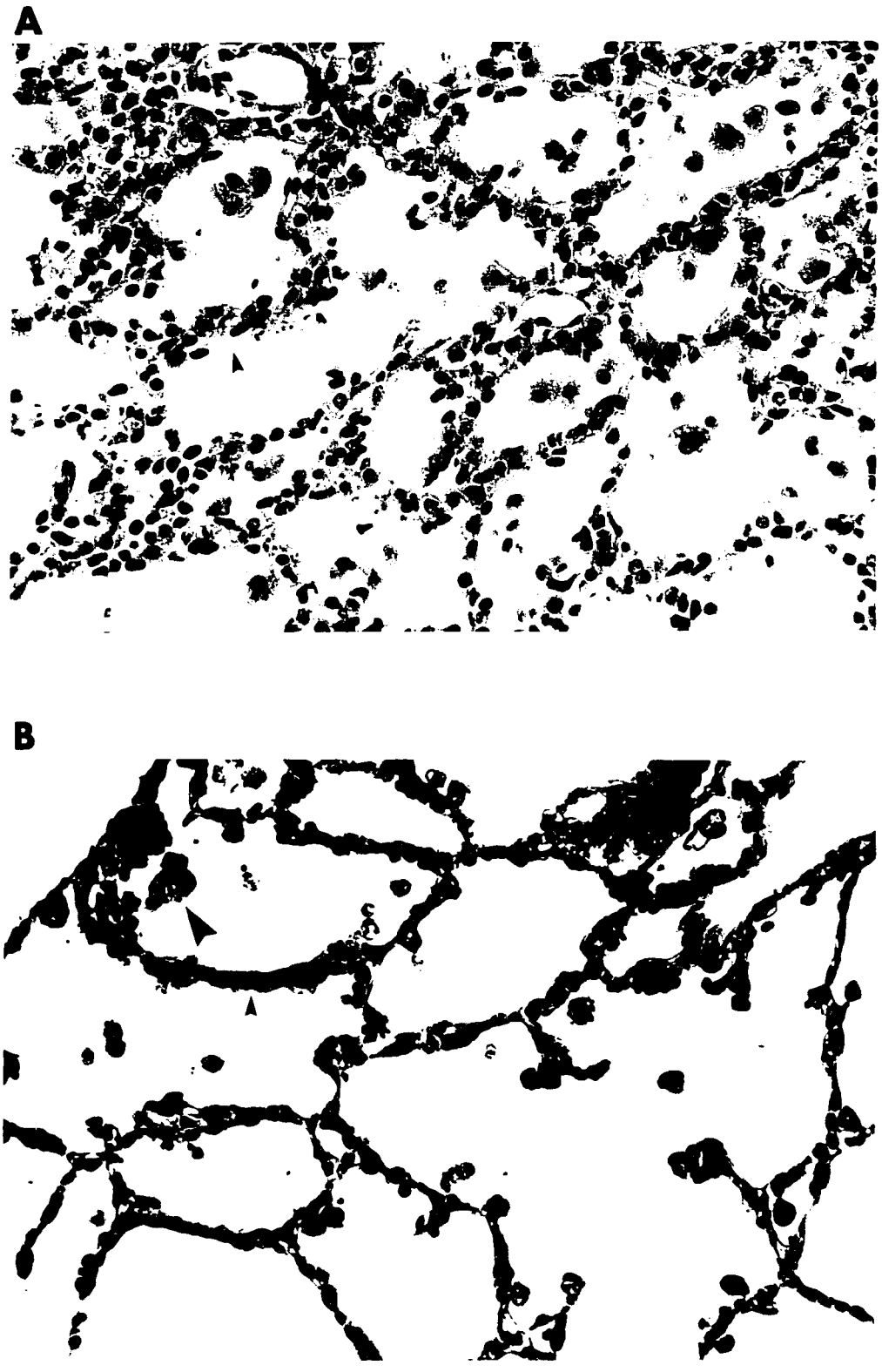

A
B

Figure 5. A. Photomicrograph of lung section of room air ureaplasma-inoculated pup that was killed by the dam 24 hours after inoculation. Neutrophils and macrophages are seen within the alveoli (arrows). B. Photomicrograph of lung section of oxygen exposed ureaplasma-inoculated pup that died 12 hours after inoculation. The most impressive feature of this section is the hemorrhage (arrows) and protein exudation in the alveoli.

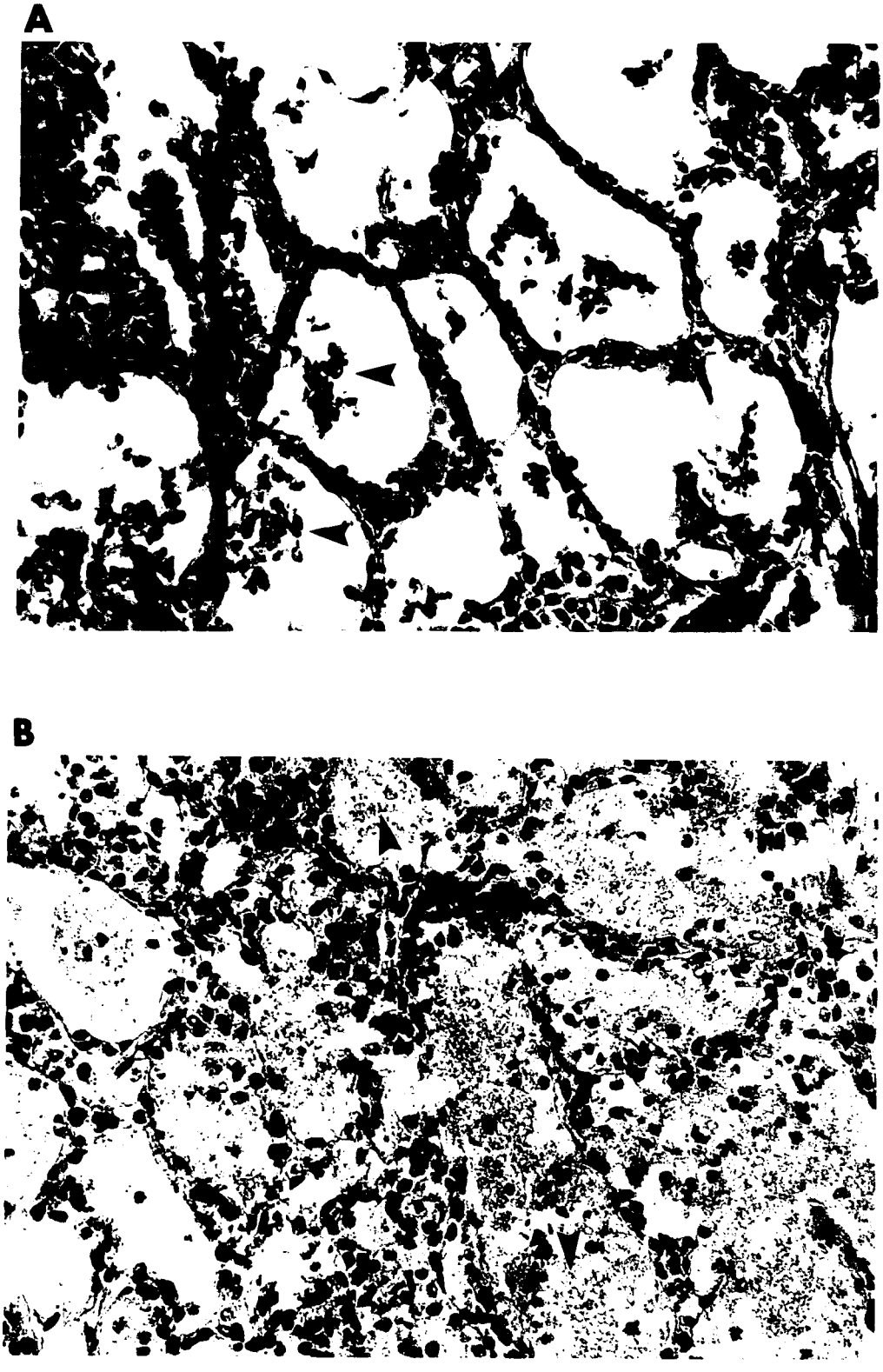
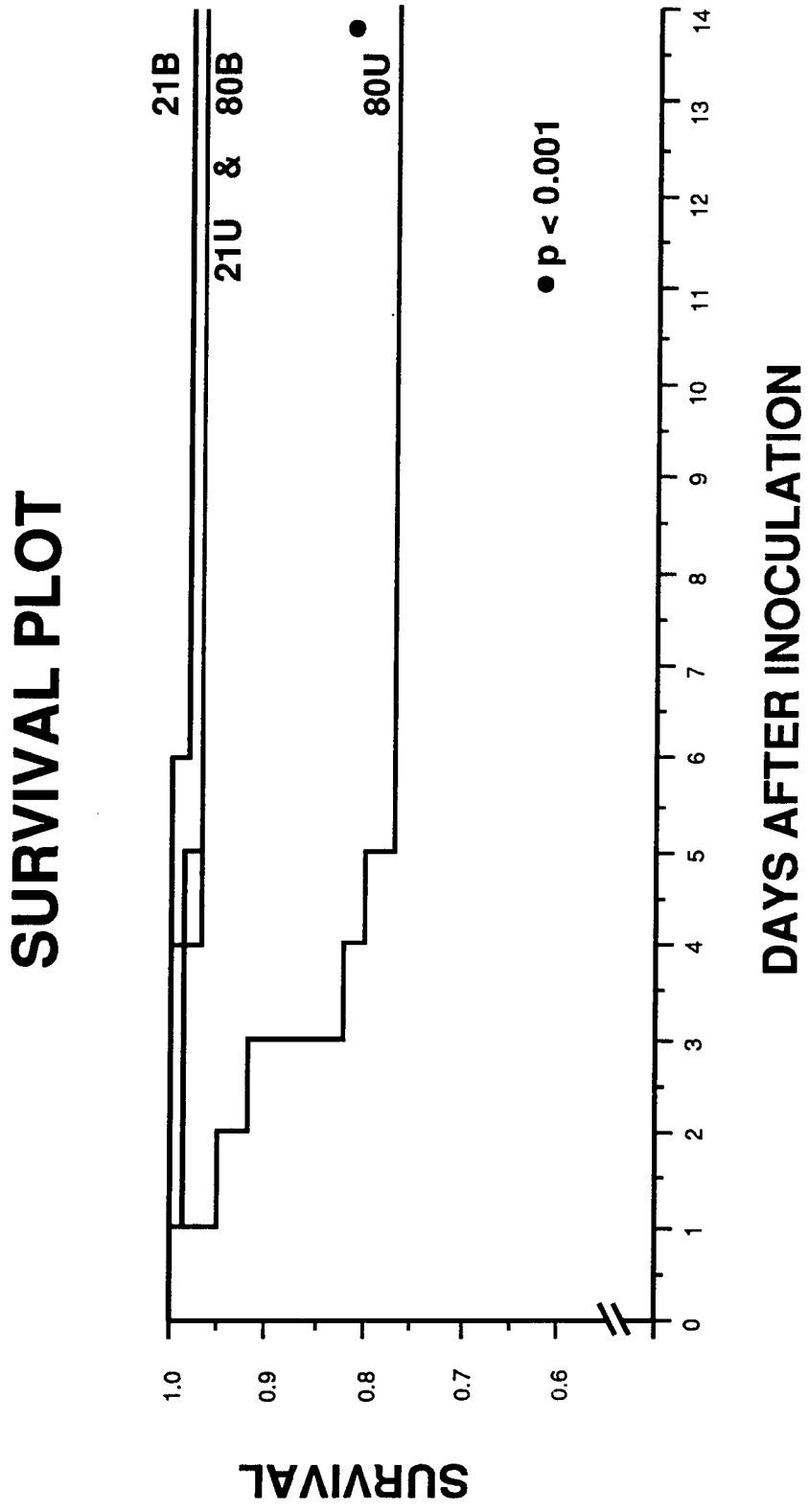

A
B

FIGURE 6. Kaplan-Meier survival plot for the four study groups. The oxygen exposed pups inoculated with U. urealyticum had significantly more mortality than any of the other study groups ($p < 0.001$, Log Rank Test).


SURVIVAL PLOT
21B
21U & 80B
80U
• p < 0.001
SURVIVAL
1.0
0.9
0.8
0.7
0.6
0
1
2
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4
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DAYS AFTER INOCULATION

AGGREVATION OF UREAPLASMA PNEUMONIA IN ADULT AND JUVENILE MICE BY HYPEROXIA

by

CROUSE, DT., CASSELL, GH., BULGER, A., AND ROLLINS, C.

Infection and Immunity (submitted)

ABSTRACT

We showed that Ureaplasma urealyticum is associated with chronic lung disease and death in very low birth weight infants, as well as with neonatal pneumonia. We developed a newborn mouse model of U. urealyticum pneumonia which fulfilled Koch's postulates. We also demonstrated that hyperoxia potentiates lung injury in this model. Based on the disease potentiation seen with hyperoxia in the newborn mouse, we postulated that exposure of immunolocally juvenile (10-12 days old) and mature mice to hyperoxia would make them more susceptible to infection. Our goal was to produce a larger animal model of ureaplasma pneumonia.

Adult or juvenile mice were exposed for 48 hours to 80% oxygen, challanged intranasally with U. urealyticum, and replaced in the hyperoxic environment. Control mice were kept in room air. Oxygen-exposed mice had significantly more organisms isolated during the 4 days postinoculation. A higher percentage of mice exposed to hyperoxia, compared to control mice, were infected at any time point during the 4 days after inoculation. The pulmonary

lesions consisted of alveolar macrophages, occasional neutrophils, proteinaceous debris, and alveolar thickening. Mice exposed to hyperoxia had more severe and persistent lesions than the control mice. Focal hemorrhage was seen occasionally in mice exposed to hyperoxia. Juvenile mice took only slightly longer to recover than the adult mice. Thus, hyperoxia diminished the pulmonary defenses of juvenile and mature mice, making them more susceptible to pulmonary infection by U. urealyticum.

INTRODUCTION

Ureaplasma urealyticum is a genital mycoplasma found in over one-half of women of child-bearing age (1,11). Until recently little data were available to implicate this microbe in perinatal infectious processes. We have shown that U. urealyticum can produce pneumonia in newborn infants, and that it is associated with chronic lung disease and death in premature infants (5,26). A newborn mouse model of U. urealyticum pneumonia was developed using an isolate from an infant with chronic lung disease to allow the study of this infection under controlled conditions (17). The purpose of the model was to provide a system to study the interaction of U. urealyticum with pulmonary host defenses, and to fulfill Koch's

postulates. Both goals were achieved. Ureaplasma pneumonia produced in newborn mice is similar histologically to that observed in human infants infected wtih this organism (4,26). The neonatal murine pulmonary lesions were characterized by a mononuclear infiltrate with some neutrophils present. This pneumonia was transient, peaked at 3 to 4 days, and resolved by approximately one week (17). Older mice were resistant to pulmonary infection with this microorganism.

We used this newborn mouse model to study the effects of hyperoxia on the ability of the newborn mouse to resist ureaplasma pneumonia (7). Hyperoxia had severe consequences on the outcome, causing increased organism persistence, increased lesion severity, and death in newborn mice infected with U. urealyticum (7). We concluded that high ambient oxygen concentrations impair the host's pulmonary defense mechanisms, thus potentiating the disease process. Based on this conclusion, we then hypothesized that hyperoxic exposure would interfere with the pulmonary host defense mechanisms in older mice and make them more susceptible to U. urealyticum pneumonia.

MATERIALS AND METHODS

Animals Pregnant pathogen-free C3H/HeN mice (dams) at 14-16 days gestation were obtained from Charles Rivers Breeding Laboratories, Raleigh, North Carolina. This colony is surveyed routinely for all known pathogenic murine mycoplasmas, bacteria, and viruses. These animals were shipped in cartons with microbial filters to UAB, where they were transferred in a laminar flow hood (The Baker Company,Sanford, ME) to sterile microisolator® filter-topped cages (Lab Products Inc., Maywood, NJ). Sterile food (Agway, Inc., Syracuse, N.Y.), water, and non-aromatic hardwood chip bedding (P.J. Murphy Forest Products, Rochelle Park, N.J.) were placed in each cage. The dams were allowed to deliver, and the pups were either used at 10 - 12 days of age (juveniles) or allowed to mature into adults (> 2 months of age) before being used for these studies. Microbial surveys for known pathogenic murine mycoplasmas, bacteria, parasites, and viruses were performed on selected mice from each experiment, and the results were negative.

These studies were approved by the Animal Use Committee at UAB and conformed to the policies set forth in the Public Health

Service NIH publication No. 86-23 "Guide for the Care and Use of Laboratory Animals."

Organism Ureaplasma urealyticum serotype 10 was obtained from an infant with refractory apnea and pneumonia who later developed bronchopulmonary dysplasia (5). This microorganism was grown in 10B medium without yeast extract, concentrated by centrifugation (14,000 x g for 40 minutes), and resuspended to 2 x 10^8 colony forming units (CFU)/ ml in 10B medium without yeast extract. The organisms were then aliquoted into 1.0 ml freezer vials and frozen at -70 °C.

Study Protocol Sixteen adult mice were divided into two groups. Eight mice were placed into a sterile plexiglas exposure cage with an humidified 80% oxygen environment. The other eight mice were placed into a similar Plexiglas exposure cage, but with an humidified 21% oxygen (room air) environment. Humidified 21% oxygen was provided to the plexiglas cage by bubbling room air from a hospital grade compressor (Timeter Instrument Corp., Lancaster, PA) through a hospital grade humidifier (Aquapak, Hudson Respiratory Care Inc., Temecula, CA) at 6 liters/minute. Humidified 80% oxygen was supplied by blending hospital-grade oxygen from a

liquid oxygen tank with room air and bubbling the mixture through a humidifier as for the 21% oxygen. The oxygen concentration was measured (5577 Oxygen analyzer, Hudson, Ventronics Division, Temecula, CA) and recorded every 12 hours while the study was in progress. Humidity, ammonia, and CO_2 were measured daily (Gas detector SG4010, Union Carbide, Linde Division, Post Welding Supply, Birmingham, AL) and kept at approximately 50% relative humidity, < 15 ppm of ammonia, and < 5% CO_2 (3). Urine and stool dropped through the 0.25 inch mesh flooring onto a sterile absorbant pad which was changed daily. Sterile food and water was supplied ad libitum. Sterile non-aromatic aspen shavings (Northeastern Products, Warrensburg, N.Y.) were used for bedding in the plexiglas cages because of the mesh flooring.

After 48 hours of exposure to the respective environments, the mice were anestetized with an intraperitoneal injection of pentobarbital; a U. urealyticum aliquot was thawed and brought to room temperature, and the mice were inoculated intranasally with 40 µl of the microorganism suspension. The mice were placed back into their respective oxygen environments and allowed to recover. At 24 hour intervals, two mice selected from each group were

sacrificed. Bronchoalveoalr lavages with 10B medium were performed on each mouse and then the lungs were removed for histology.

The same protocol was used for experiments with the juvenile mice with the following exceptions. Juvenile mice were housed in litters along with a dam since they had not been weaned. The mice were sacrificed at 4-5 days after inoculation.

Culture Methods All 10B medium used in these experiments was prepared without yeast estract. Bronchoalveolar lavages with 10B medium were performed immediately after sacrifice. The lavage was serially 10-fold diluted to 10^{-6} in 10B medium and plated on A8 agar plates. The broth dilutions were incubated at 37°C in room air, whereas the agar plates were incubated at 37°C in 5% CO_2. The dilutions and plates were kept for 7 days before being discarded as negative.

Histology The lungs of the adult mice were removed and inflated at 20 cm H_2O pressure with 95% cold ethanol for at least 30 minutes. The tracheas were then ligated and the lungs were fixed in 95% cold ethanol for at least one week. The lungs were then separated into their corresponding lobes, imbedded in parafin,

sectioned, and stained with hematoxylin and eosin. The lungs of juvenile mice were removed and inflated with cold 95% ethanol until the acute angles were distended (7). The lungs were then immersed in 95% cold ethanol for at least a week before sectioning. The sections were then handled in the same manner as the adult lungs.

Statistics Culture data were log transformed prior to being analyzed. The Fisher's Exact Probability Test was used to analyze the proportional data, and the Student's t-Test was used to analyze the log-transformed culture data. The level of significance was arbitrarily set at $p < 0.05$. All statistics were performed with the Statistica Mac software package (Statsoft, Tulsa, OK).

RESULTS

Culture Results One adult mouse died during inoculation, but no deaths were seen during the environmental exposure period in this study. The adult mice in the hyperoxic ($n = 30$) environment had over a four-fold greater number of mice with positive cultures for *U. urealyticum*, compared to the normoxic group ($n = 29$) ($p = 0.0003$, Fisher's Exact Probability Test) (Fig. 1A). The number of organisms per mouse was also significantly greater in the hyperoxic group ($p < 0.0001$, Student's t-Test) (Fig. 1B). Important to note is the

distribution of positive cultures over the 96 hour time period (Fig. 2). Most adult mice in the normoxic group were able to clear U. urealyticum from their lungs within 24 hours of inoculation, compared to mice in the hyperoxic group which still had positive cultures out to 96 hours.

Similar results were noted in the experiments with juvenile mice, (Fig. 1A and B). More juvenile mice in the hyperoxic group (n = 27) were culture positive when compared to those in the normoxic group (n = 26) ($p = 0.002$, Fisher's Exact Probability Test) (Fig. 1A). Similarly, the number of microorganisms was greater in the juvenile mice in the hyperoxic group when compared to mice in the normoxic group ($p < 0.003$, Student's t-Test) (Fig. 1B).

Histology Data Twenty-four hours after inoculation, adult mice in the room air group had a mild alveolar infiltrate which consisted mainly of alveolar macrophages (Fig. 3A and B). Usually fewer than two cells were found free within a given alveolus. Little proteinaceous debris was found within the alveolar spaces or airways and the alveolar walls were only slightly thickened. In contrast, adult mice in the hyperoxic group were more severely affected (Fig. 4A and B). Many of the alveolar spaces were

atelectatic whereas others were overdistended (Fig. 4A). The adult mice in the hyperoxic group had a more severe alveolar infiltrate which consisted mainly of alveolar macrophages and some neutrophils (Fig. 4B). More proteinaceous debris was present and the alveolar walls were thicker and had increased cellularity. Occasionally, focal hemorrhage was found in the lung sections from adult mice in the hyperoxic group, but not from mice in the normoxic group (Fig. 5).

Forty-eight hours after inoculation, adult mice in the normoxic group had minimal evidence for pulmonary disease compared to adult mice in the hyperoxic group (Fig. 6A and B). Alveolar macrophages were seldom seen in the histologic sections from the normoxic group, whereas numerous alveolar macrophages were present in the section from the hyperoxic group. The alveolar walls were also noticeably thicker in the hyperoxic group compared to the normoxic group. Few, if any, neutrophils were present in sections from either group.

By 96 hours after inoculation, the pulmonary lesions in both groups had almost disappeared. Little difference between the pulmonary lesions in the normoxic group, compared to the hyperoxic

group, could be seen. The alveoli in the hyperoxic group were distended and not atelectatic. Very few cells and little debris remained in the alveolar spaces of the hyperoxic group. The alveolar walls still seemed to be slightly more cellular than those from the normoxic group.

Histological sections from the juvenile mice demonstrated similar findings (Fig. 7A and B). The predominate inflammatory cell was the alveolar macrophage (Fig. 7B). The alveolar walls appeared to be slightly thicker and more cellular in the hyperoxic group compared to the normoxic group. Proteinaceous debris was also more prevalent in the sections from the hyperoxic group.

DISCUSSION

These results demonstrate that high concentrations of oxygen interfere with normal pulmonary defense mechanisms and allow U. urealyticum to produce a pneumonia in mice which are usually resistant to this organism. By histological examination, adult mice exposed to 80% oxygen recovered almost completely from pneumonia within 96 hours. Conversely, by culture data, almost half of the adult mice were still infected at 96 hours. Juvenile mice exposed to hyperoxia took slightly longer to recover, as pneumonia was still

present at 96 hours and over half of the mice were culture positive. The pulmonary lesions were more severe in mice exposed to hyperoxia compared to mice in the normoxic group. The pulmonary lesions in mice exposed to 80% oxygen consisted of alveolar macrophages with occasional neutrophils, proteinaceous debris, alveolar thickening, and occasional hemorrhage. All animals survived the experimental pneumonia and hyperoxic exposure, a fact which is in contrast to our previous results with newborn mice (7).

Interesting to note was the fact that in the adult mice the inflammatory infiltrate was resolving while ureaplasmas still existed in the respiratory tract. Ureaplasmas are known to infect ciliated respiratory epithelium and cause ciliostasis, as well as to infect the alveolar spaces (15). No inflammatory infiltrate was detected in the airways of any of the mice in this study. In theory, the tracheobronchial tree could act as a reservoir for future alveolar infection or antigen exposure if ureaplasmas persisted on the respiratory epithelium.

U. urealyticum infected mice with mature, or maturing, pulmonary defense mechanisms are not as severely affected by hyperoxic envirionments as are neonatal mice. The persistence of

organisms in neonatal mice may be due to immature pulmonary defense mechanisms, increased susceptibility to oxygen, or both. In our previous study, the pulmonary defense mechanisms of neonatal mice were severely affected by exposure to 80% oxygen (7). U. urealyticum persisted in the lungs for up to 2 weeks in neonatal mice exposed to the hyperoxic environment, whereas neonatal mice in the normoxic environment cleared the microorganisms. The pulmonary lesions in neonatal mice consisted of the same elements present in the lesions of the adult mice, but were more severe. No death was produced in the adult or juvenile mice in this study, whereas we demonstrated approximately 24 % death in neonatal mice with ureaplasma pneumonia subjected to hyperoxia. Thus, not only are more mature mice more resistant to pulmonary infection from U. urealyticum than neonatal mice, even in hyperoxic conditions, but they are able to recover from the infection quicker.

Our results are in accordance with previous reports with other microbes. Exposure to nitrogen dioxide, another oxidant, increases the susceptibility of mice to Mycoplasma pulmonis and decreases the clearance of these microbes from the respiratory tract (14). Hyperoxia adversely affects the pulmonary clearance of

Pseudomonas aeruginosa in mice, possibly by reducing the influx of polymorphoinuclear leukocytes into the alveolar spaces (8).

Older animals and humans are usually much more resistant to infection than their young (2,6). Several defense mechanisms are not mature in the neonate, which makes them more susceptible to infection (10). Neonatal macrophages may have normal phagocytic abilities, but are deficient in the production of gamma-interferron and microbial killing by the oxygen-dependent and oxygen-independent antimicrobial systems (13,19,22,24,27). Neonatal neutrophils also have defects in chemotaxis and intracellular killing (12,20). In addition, hyperoxia inhibits chemotaxis, phagocytosis, and the oxygen-dependent antimicrobial system (9,16,21,23). Thus, neonatal macrophages and neutrophils which may be functioning poorly may be impaired futher by hyperoxic environments.

Preterm human infants are much more susceptible to infection than term infants (25). This is due to the additional immunological defects seen in preterm human and animal neonates. Sherman et al. has demonstrated that preterm rabbits cannot clear group B streptococci from their lungs as well as those studied at term (18). Preterm rabbits have fewer alveolar macrophages than term rabbits

and must rely on neutrophil recruitment to fight group B streptococcal pulmonary infection (28). Thus, preterm animals, including human infants, would likely be more severely handicapped by any modality that adversely affects pulmonary clearance, such as hyperoxia. Importantly, our study demonstrates that even animals with mature pulmonary defenses can be adversely affected by a hyperoxic environment.

This model serves many purposes. These data further confirm that ambient hyperoxic environments can be detrimental to pulmonary defenses. This effect may be more pronounced in immature animals and humans, but still is present in more mature hosts. These experiments show that adult animals exposed to hyperoxia can be used as a model to study U. urealyticum pneumonia. Pulmonary defenses against U. urealyticum which could not be studied in newborn mice can now be accomplished. This model will not be appropriate for the study of barotrauma, another factor in chronic lung disease in human neonates, because of the size of the host. A larger animal of U. urealyticum pneumonia which can be ventilated will need to be developed to study the effects of pressure. Overall, we have demonstrated with an animal model that

U. urealyticum can cause pneumonia, and that hyperoxic conditions exacerbate the respiratory disease produced. These observations are consistent with our understanding of the human neonatal disease process. The efficacy of various treatments can be studied in the neonatal and adult animal models, and will possibly give direction to future treatment trials in human neonates.

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Fig. 1.A. Percentages of U. urealyticum-inoculated adult and juvenile mice that were culture positive after exposure to either room air or hyperoxia. Data from all time points were summed in the adult groups. Significantly more mice exposed to 80% oxygen were culture positive compared to those in room air: adult - $p = 0.0003$, juvenile - $p = 0.002$, Fisher's Exact Probability Test. B. Mean number of colony fouming units of U. urealyticum isolated per mouse. Error bars represent plus one standard deviation. Significantly more organisms were isolated from the mice exposed to 80% oxygen compared to those in room air: adult - $p < 0.0001$, juvenile - $p < 0.003$, Student's t-test.

Figure 1B.

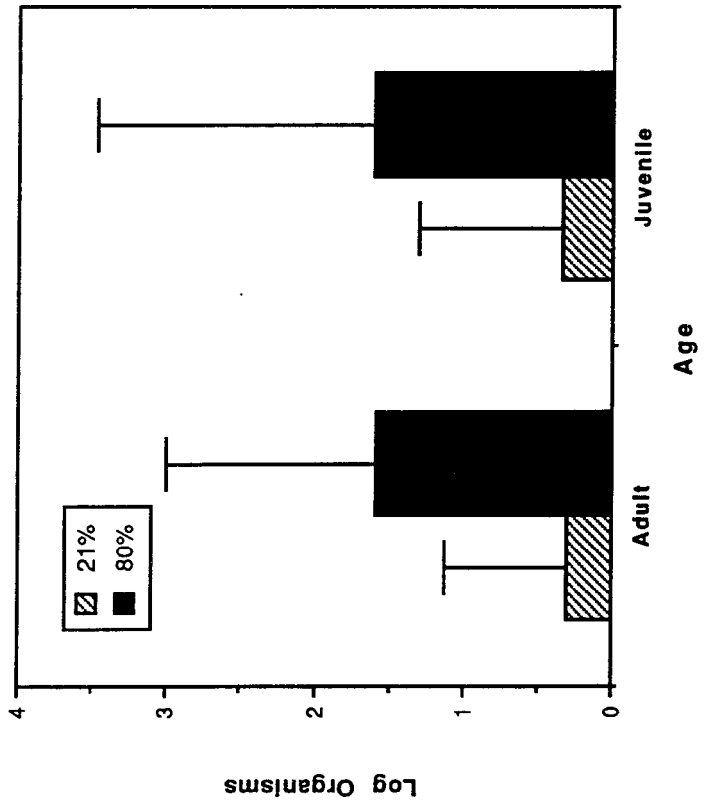


Figure 1A.

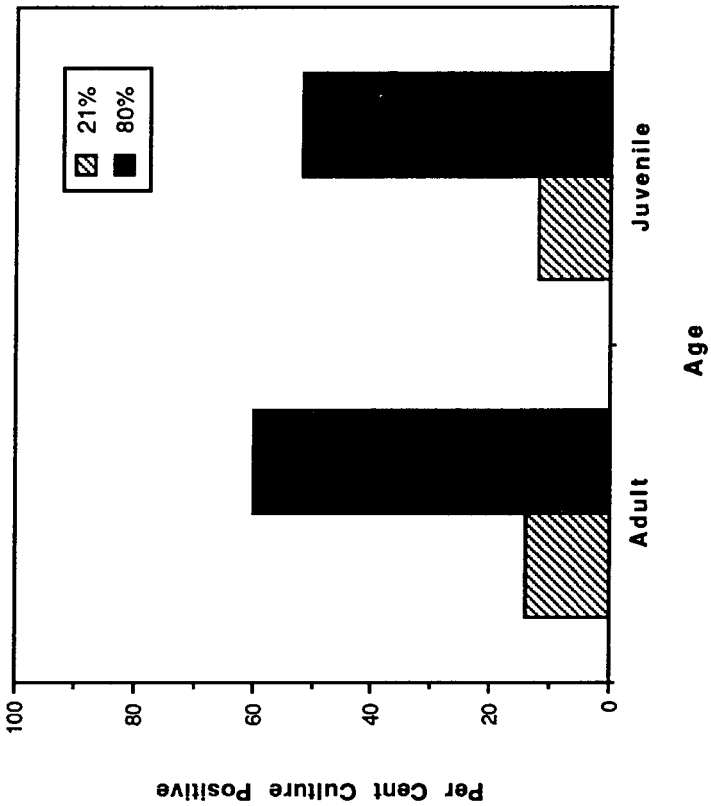


Figure 2.

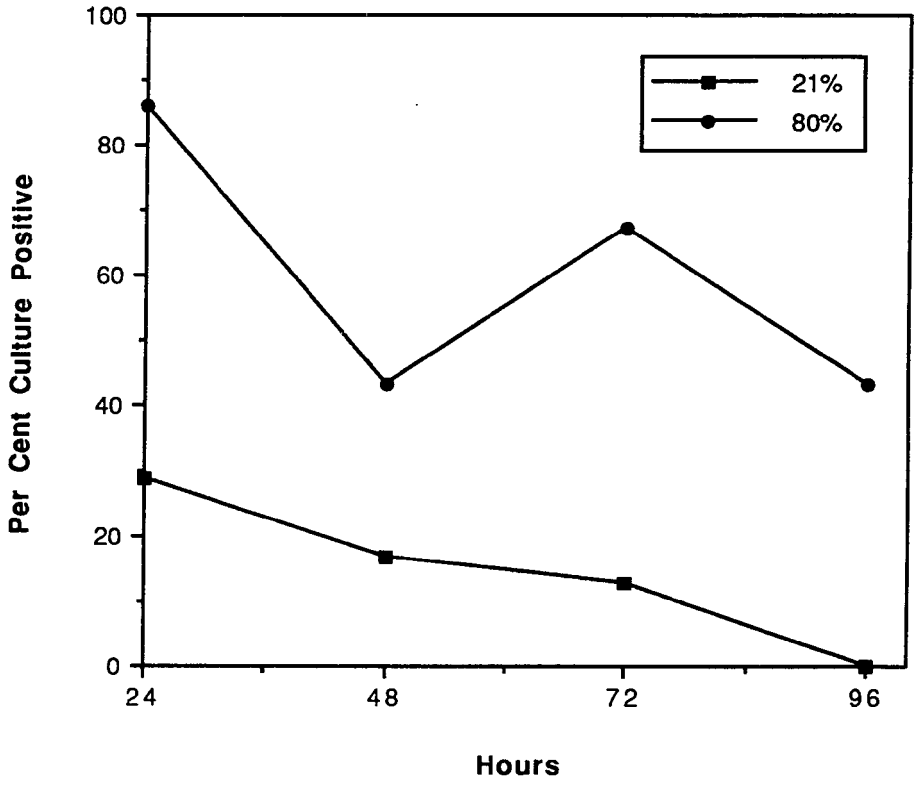
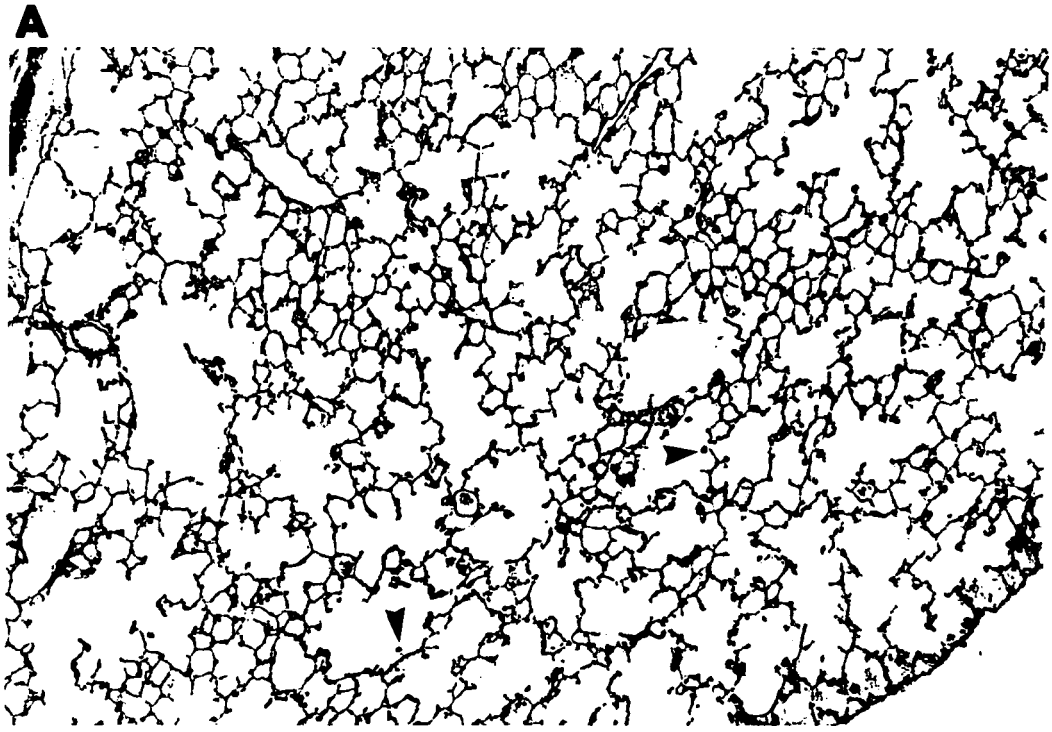


Fig. 2. Percentages of U. urealyticum-inoculated adult mice that were culture positive in each oxygen concentration at individual culture time points. The percentage of culture positive adult mice at each time point was greater in the 80% oxygen exposed group compared to the room air group.

Fig. 3. Photomicrographs of lungs from adult mice inoculated with U. urealyticum and placed in room air for 24 hours. Lungs were perfused with 95% cold ethanol at 20 cm H_2O pressure. A. Low power photomicrograph (40x) showing relatively normal alveolar size with an occasional free alveolar macrophage (arrow). B. High power photomicrograph (100x) showing alveolar macrophages (arrows) and some proteinaceous debris. Alveolar walls are only slightly thickened.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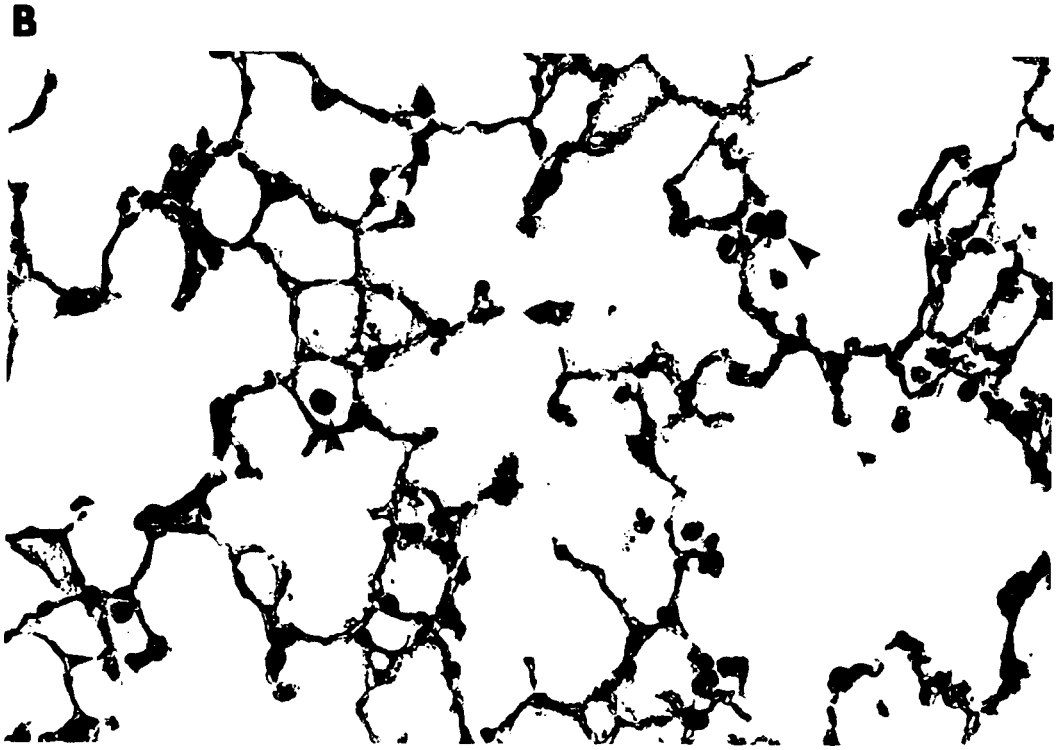

B

Fig. 4. Photomicrographs of lungs from adult mice inoculated with U. urealyticum and exposed to 80% oxygen for 24 hours. Lungs were perfused with 95% cold ethanol at 20 cm H_2O pressure. A. Low power photomicrograph (40x) showing over distended alveoli interspersed with ateletatic alveoli (small arrows). Notice the vasodialtion caused from exposure to hyperoxia (large arrow). B. High power photomicrograph (100x) showing alveolar accumulations of foamy macrophages (small arrows) with occasional neutrophils. Proteinaceous debris is scattered through the alveoli. The alveolar walls are thickened by increased cellularity (large arrows).

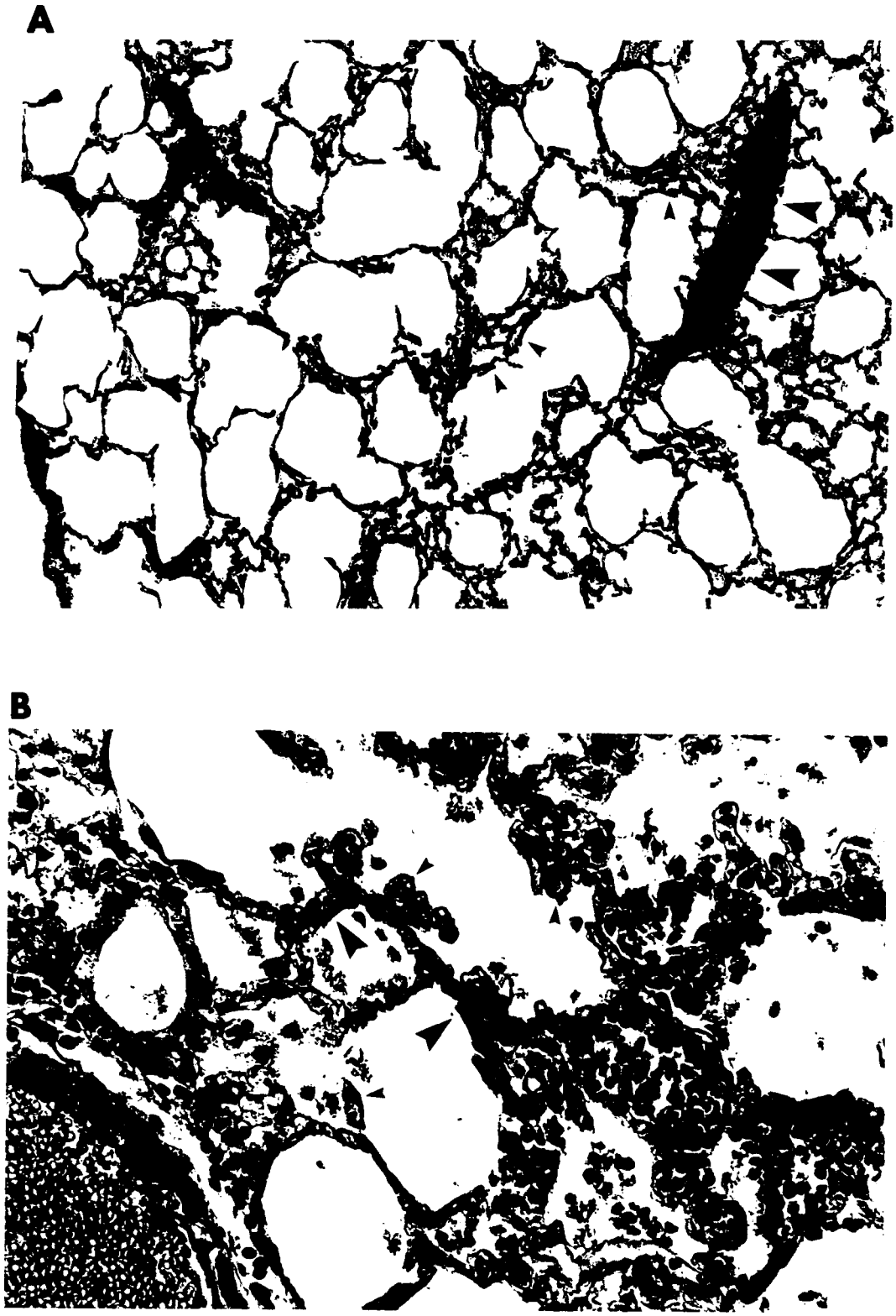

A
B

Fig. 5. Photomicrograph of lung section from an adult mouse inoculated with U. urealyticum and exposed to 80% oxygen for 24 hours (40x). This section demonstrates focal areas of hemorrhage (small arrows) that were found occasionally in the hyperoxia exposed mice and never seen in the room air exposed mice.

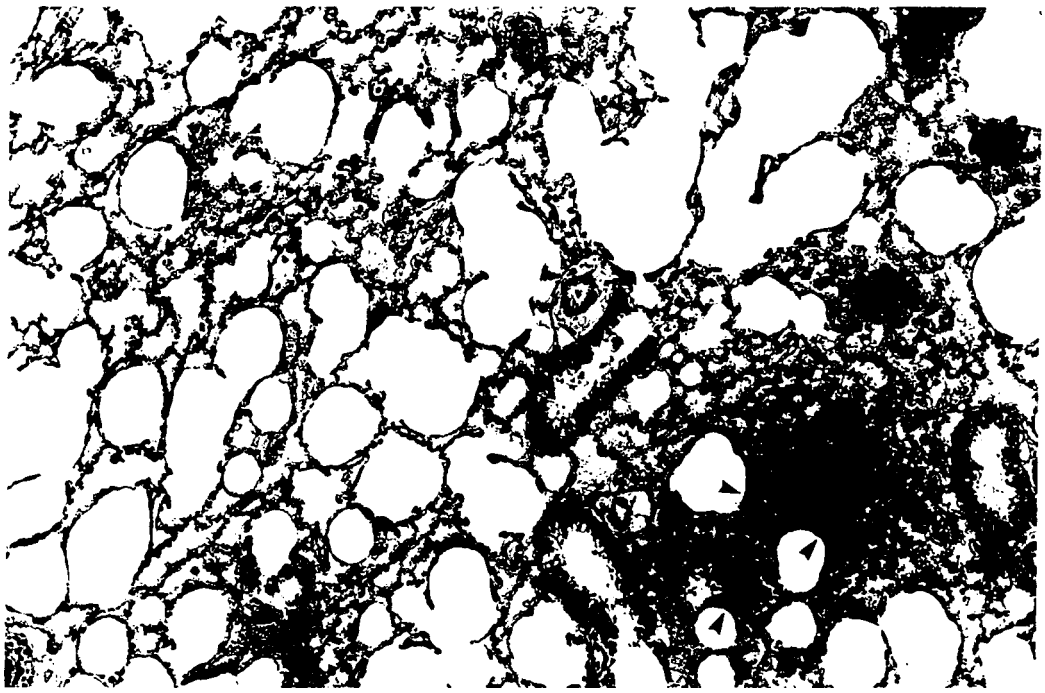


Fig. 6. Photomicrographs (100x) of lungs from adult mice inoculated with U. urealyticum and exposed to either room air or 80% oxygen for 48 hours, (A. and B., respectively). The alveolar walls the room air exposed mice are thin with only occasional areas of thickening, (arrow). Few alveolar macrophages are present, (small arrow). In contrast, the alveolar walls of mice exposed to hyperoxia are much thicker (arrows) and the alveoli are not as well distended. Proteinaceous debris is still quite prevalent as are foamy macrophages (small arrows).

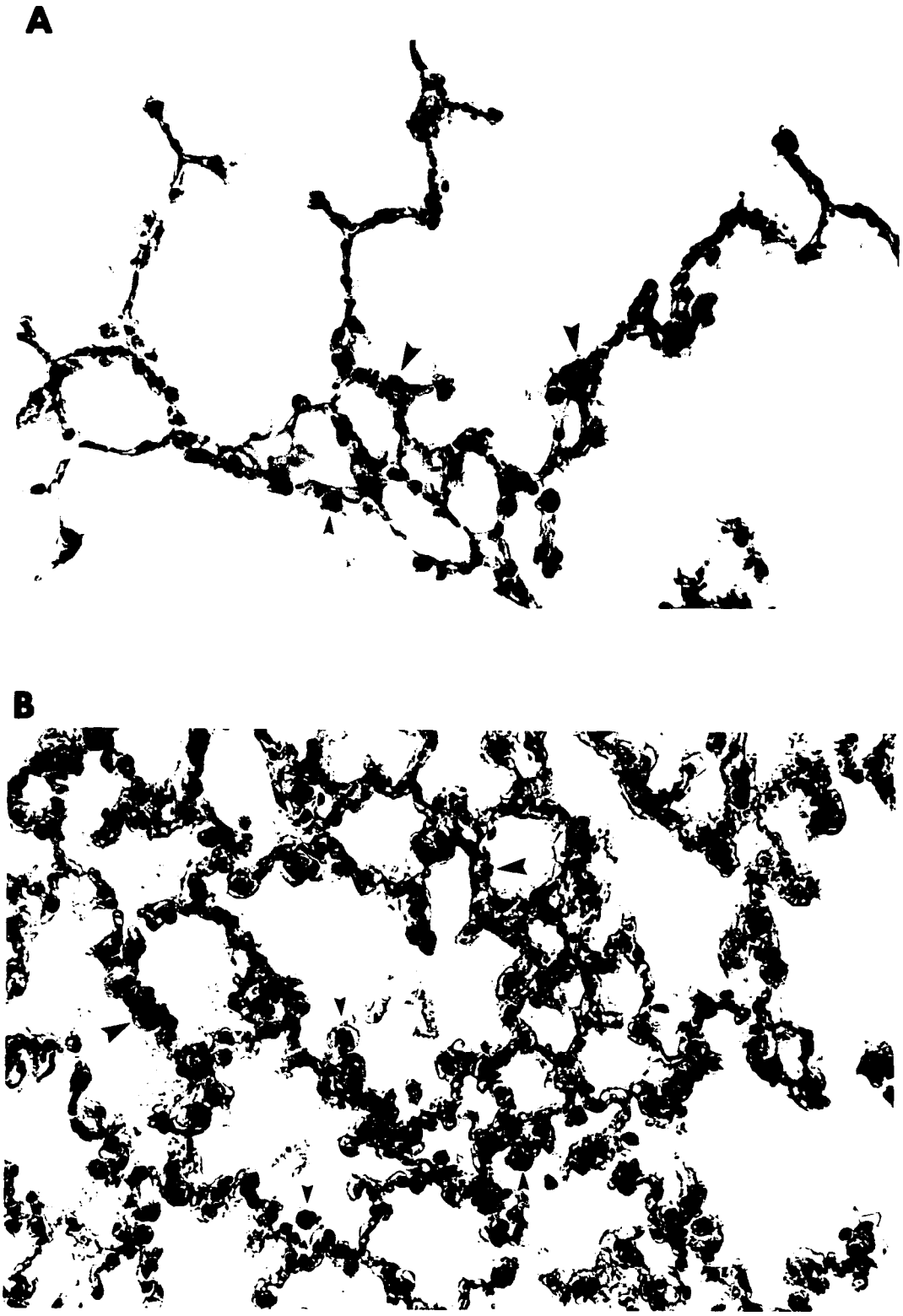
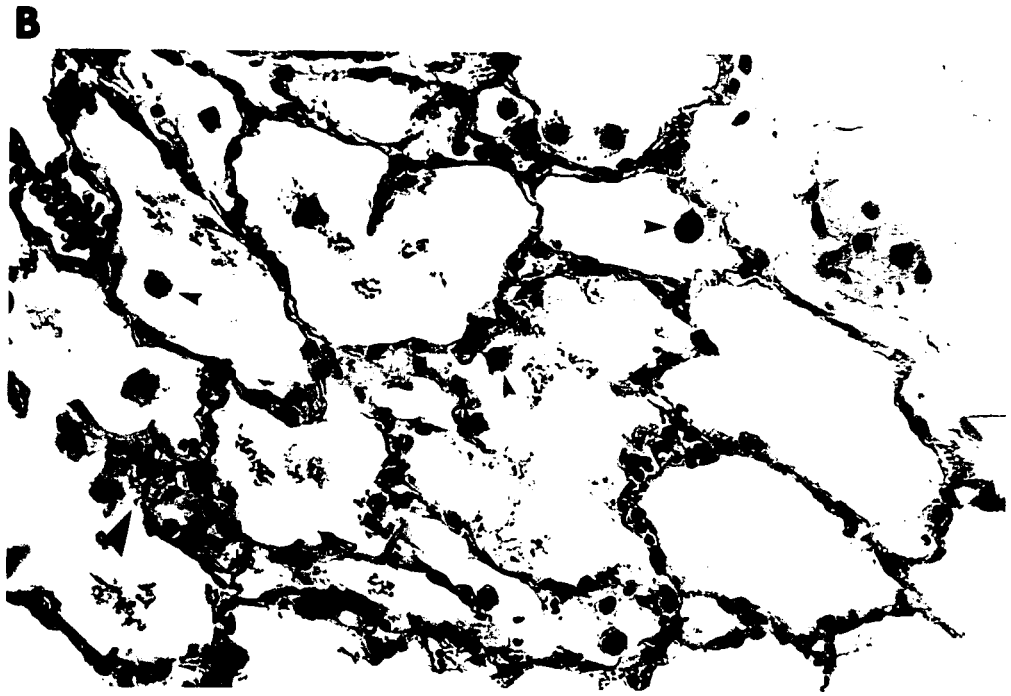

A
B

Fig. 7. Photomicrographs (100x) of lungs from juvenile mice inocuated with U. urealyticum and exposed to either room air of 80% oxygen for 96 hours, (A. and B., respectively). More alveolar macrophages were present in the lungs of mice exposed to hyperoxia than those exposed to room air (arrows). In fact, some alveoli were almost filled with phagocytic cells (large arrows). In addition, slightly more debris was present in the hyperoxic group.


A


B

SUSCEPTIBILITY OF UREAPLASMA UREALYTICUM AND MYCOPLASMA HOMINIS TO OXIDANT-MEDIATED DAMAGE IN VITRO

by

CROUSE, DT. AND BULGER, A.

Infection and Immunity (Submitted)

ABSTRACT

We investigated the effects of oxidants on growth of Ureaplasma urealyticum and Mycoplasma hominis in vitro. These microorganisms were incubated with various concentrations of H_2O_2 for up to 4 hours. Iron or iron plus iodine were added to some experiments to produce the hydroxyl radical (·OH) or ·OH and a hypohalide (HOI), respectively. Both microbes were relatively resistant to H_2O_2. The addition of Fe or Fe+I increased the growth inhibition of both microorganisms. In a separate set of experiments, U. urealyticum and M. hominis were incubated with the catalase inhibitor, aminotriazole, to test whether catalase contributes to protection from oxidant injury. Aminotriazole did not affect the growth of either microorganism in the absence of oxidants. U. urealyticum could tolerate approximately one log greater mM concentration of H_2O_2 compared to untreated organisms. Conversely, M. hominis was more susceptible to oxidant injury. The growth inhibition of M. hominis by aminotriazole was demonstrated only in conditions where the ·OH was produced. Thus, catalase is likely required for protection from oxidants in M. hominis but not in U. urealyticum. The probable mechanism operative in protection of M. hominis by catalase is to prevent the formation of ·OH or ·OH and HOI.

INTRODUCTION

Eradication of microorganisms from the lower respiratory tract involves the coordinated effort of many mechanisms (18). One important component of these pulmonary defense mechanisms is phagocytosis and killing of microbes by phagocytic cells (8). Oxygen-dependent killing by phagocytes is mediated by the elaboration of toxic oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) (2,10,15). Production of these oxygen metabolites is characterized by increased oxygen uptake into the phagocytic cell, a process known as the respiratory burst (2).

Reaction of ·OH with a halide such as chlorine or iodine (I) produces a hypohalide, or halide radical, which is extremely toxic to living cells (13). Neutrophils and monocytes contain myeloperoxidase which catalyzes this reaction within the phagolysosome. Alveolar macrophages, the primary resident phagocyte in the lower respiratory tract, contain very little, if any, myeloperoxidase. Instead, iron (Fe) is thought to catalyze the necessary reaction to produce a hypohalide within the phagolysosome (9,26). In some instances, free eosinophil peroxidase or myeloperoxidase within the inflammatory infiltrate can attach to microorganisms and be phagocytized by macrophages to provide the necessary catalyst to produce a hypohalide (15,21). Although chlorine is the most common halide present in the phagolysosome, iodine also can be used and the resultant iodide radical (·I) is toxic to microorganisms (14,16).

Yamada et al. has used the H_2O_2-Fe-Halide system to demonstrate susceptibility of various microorganisms to oxidative killing and also has shown that inhibitors of ·OH production greatly diminish microbial killing in this system (30). In this study, we used the H_2O_2-Fe-Halide system to demonstrate the sensitivity of Ureaplasma urealyticum and Mycoplasma hominis to oxidant injury. These organisms are frequently found in the respiratory tract of very premature infants who are receiving supplemental oxygen, leading us to question the susceptibility of these bacteria to oxidant injury (4). U. urealyticum can persist for months in the respiratory tracts of infants who are on high amounts of oxygen (4). Hyperoxia increased organism persistence, lesion severity, and death in a mouse model of U. urealyticum pneumonia (6). M. hominis contains catalase which might protect it from oxidant injury (19). Based on these data, we hypothesized that U. urealyticum and M. hominis would be resistant to oxidant mediated damage, since these microbes can persist in a high oxygen environment or produce catalase which catalyzes the breakdown of H_2O_2 to H_2O and O_2.

MATERIALS AND METHODS

Reagents All reagents were purchased from Sigma Chemical Company, St. Louis, MO. Stock 20 mM solutions of ferrous sulfate, sodium iodide, and EDTA were prepared fresh the day of experimentation. Also prepared fresh each day was 1.0 M Aminotriazole. Minimum essential media (MEM #3193, Sigma) without sodium bicarbonate or phenol red were prepared and the pH was adjusted to 6.95. Hydrogen peroxide, 30% by weight, was diluted and

assayed daily at 240 nm (3). A 1.0 M solution of H_2O_2 was prepared and serial log 10 dilutions were made. All reagents were prepared with endotoxin-free ultrafiltered deionized water with a resistance of at least 18 megohm x cm (Milli-Q UF, Millipore, Bedford, MA).

Microorganisms U. urealyticum (serotype 10) was originally obtained from the respiratory tract of an infant in the Regional Newborn Intensive Care Unit (RNICU) who had respiratory disease (4). The organism was grown in pure culture in 10B medium without yeast extract, passed through pathogen-free mice six to eight times, regrown, and concentrated to approximately 2.0 x 10^8 colony forming units (CFU)/ ml (6). Aliquots of 1.0 to 1.5 ml were frozen at -70°C until use. M. hominis was obtained from the respiratory tract of an infant with pneumonia in the RNICU, grown in pure culture in 10B medium with yeast extract, divided into 1.0 to 1.5 ml aliquots, and frozen at -70°C until use. The average concentration of M. hominis in the frozen stocks was 2.0 x 10^5 cfu/ml.

Media Effect on Survival Stocks of U. urealyticum and M. hominis were thawed at room temperature. Two hundred microliters of U. urealyticum stock were added to 1.8 ml of 10B medium without yeast extract (10B-YE) and to 1.8 ml of MEM. The same was done with M. hominis, except that 10B plus yeast extract (10B+YE) was used. One hundred microliter samples were taken immediately from each well. After 4 hours of incubation at 37 degrees C on a platform rocker (Bellco Biotechnology, Vineland N.J.) another 100 µl sample was obtained. The U. urealyticum samples were serially diluted in 10B-YE while the M. hominis samples were diluted in 10B+YE. Twenty microliter

samples of each dilution were spotted on A8 plates. The serial dilutions were incubated at 37 degrees C and the A8 plates were incubated at 37 degrees C in 5% CO_2. Colony forming units were determined when individual colonies could be identified on the A8 plates.

Microbial Killing Assay Stocks of the microorganisms were thawed at room temperature. A ten-fold dilution of each organism was made in MEM and allowed to stand at room temperature for one hour. The reactions were carried out in a 24 well microtiter plate (Costar, Cambridge, MA). Three rows of six wells each were filled with 1.9 mls of MEM. One hundred microliters of U. urealyticum were then added to each well. MEM (10 µl) was added to the first well of each row as a reagent control. To the second well in each row, 20 µl of 30% H_2O_2 was added. To the third through sixth wells in each row, 20 µl of successive serial log dilutions of H_2O_2 was added. No additional reagents were added to the first row. Ten microliters of ferrous sulfate and EDTA were added to each well in the second row. Ten microliters of ferrous sulfate and sodium iodide were added to each well in the third row. Immediately after all reagents were added to the wells, a 100 µl sample was taken for a baseline quantitative culture. The plates were then incubated for 4 hours at 37 degrees C on a platform rocker, after which another sample was taken for quantitative culture. The same experimental procedure was used for M. hominis.

Culture One hundred microliters of each sample were added to either 0.9 ml of 10B-YE (U. urealyticum) or 10B+YE (M. hominis). Serial ten-fold dilutions to 10^{-8} in the same medium were made. Twenty microliter samples

were plated on A8 medium for each dilution. The plates were incubated at 37 degrees C in 5% CO_2 for 7 days before discarding as negative.

Microbial killing with catalase inhibitor The microbial killing assays were carried out in the same manner as described above except that the diluted organisms were incubated for one hour with MEM and aminotriazole, 10 mM final concentration. Aminotriazole is an irreversible inhibitor of catalase (5).

Statistics All culture data were log transformed prior to analysis. Significant differences within, or between, groups were detected by the analysis of variance technique. Tukey's HSD post-hoc test was used to detect individual differences within groups. The analyses were performed with the Statistica Mac software package (Statsoft, Tulsa, OK). The level of significance was set at $p<0.05$.

RESULTS

Media Effect on Survival Table 1 presents the data from the incubation in the various media. No significant effect on survival could be demonstrated. U. urealyticum had a less than 1% loss in viability while M. hominis gained less than 2% over the 4 hour incubation at 37 degrees C. Also, no growth inhibition attributable to MEM was demonstrated in the microbial killing assay, (Fig. 1A, 1B, 2A, and 2B). No growth inhibitory effects of aminotriazole on either organism could be demonstrated over the 4 hour study period by comparing the baseline and 4 hour samples of the control (0.0 mM H_2O_2 concentration), (Fig. 3A, 3B, 4A, and 4B).

Microbial killing In these experiments U. urealyticum and M. hominis were exposed to concentrations of H_2O_2 which varied from .01 to 100 mM for up to 4 hours. A reagent control which had MEM substituted for H_2O_2 was used in each experiment. Iron was added to test for the sensitivity to ·OH and Fe+I was added to test for the sensitivity to hypohalides. U. urealyticum was relatively resistant to H_2O_2, (Fig. 1A and 1B). No significant inhibition of growth by H_2O_2 occurred in the baseline samples, whereas significant inhibition of growth in the 4 hour samples occurred in the 10 and 100 mM H_2O_2 concentrations, (Fig. 1A and 1B). Growth inhibition was not enhanced at either timepoint by the addition of Fe, (Fig. 1A and 1B). In contrast, the addition of Fe+I caused significant inhibition of growth in the 100 mM H_2O_2 baseline sample and in the 1 mM and greater H_2O_2 concentrations after 4 hours of incubation, Fig. 1A and 1B. Thus, H_2O_2 or H_2O_2+Fe had little effect on the growth of U. urealyticum, except at the highest concentrations of H_2O_2, whereas H_2O_2+Fe+I caused additional growth inhibition.

The growth characteristics of M. hominis in the presence of H_2O_2 was similar to that of U. urealyticum, (Fig. 2A and 2B). No growth inhibition occurred at any H_2O_2 concentration in the baseline samples, whereas, in the 4 hour samples significant inhibition occurred only in the highest concentration, (Fig. 2A and 2B). The addition of Fe significantly reduced the growth of M. hominis at baseline in the 100 mM H_2O_2 concentration, and at 4 hours in the 1 mM and greater H_2O_2 concentrations, (Fig. 2A and 2B). The combination of Fe+I reduced growth at each time point only slightly more than H_2O_2 alone, (Fig. 2A

and 2B). M. hominis is relatively resistant to H_2O_2 and only slightly more sensitive to H_2O_2+Fe+I. In contrast, H_2O_2+Fe produced significant growth reduction at much lower concentrations of H_2O_2.

Microbial killing with catalase inhibition These experiments were performed the same way as above except that each microorganism was preincubated in MEM containing 10 mM aminotriazole for one hour prior to experimentation. Preincubation of U. urealyticum in aminotriazole had a slight protective effect on growth, whereas preincubation of M. hominis with aminotriazole was detrimental, (Fig. 3A, 3B, 4A, and 4B). No growth inhibition of U. urealyticum occurred in any of the baseline experimental conditions, (Fig. 3A). Significant inhibition of growth occurred after 4 hours in the greatest concentration of H_2O_2, and in the greatest two concentrations of H_2O_2 when either Fe or Fe+I is added, (Fig. 3B). Approximately one log greater concentration of H_2O_2 was needed to significantly inhibit growth of U. urealyticum when these organisms were preincubated in MEM containing aminotriazole compared to MEM alone.

Preincubation of M. hominis with aminotriazole did not increase growth inhibition in the baseline samples as it did in the 4 hour samples, (Fig. 4A and 4B). At baseline, growth was only inhibited in the 100 mM H_2O_2 concentration and then only when Fe was present, (Fig. 4A). The growth of M. hominis was significantly decreased in 100 mM concentration of H_2O_2 at 4 hours, (Fig. 4B). More importantly, the growth was significantly inhibited in H_2O_2 concentrations of 0.1 mM and greater when Fe was present, and at H_2O_2 concentrations of 1.0

mM and greater when Fe+I was present, (Fig. 4B). The growth inhibition of M. hominis by H_2O_2 in the presence of Fe or Fe+I required approximately one log less dilution of H_2O_2 when these microbes were preincubated with aminotriazole, (Fig. 2B and 4B).

DISCUSSION

Our results demonstrate that both U. urealyticum and M. hominis are relatively resistant to injury from H_2O_2. The concentration of H_2O_2 needed to inhibit growth of these microbes exceeded 1.0 mM in all cases and in the majority of the experiments exceeded 10 mM. These levels of H_2O_2 are far greater than those produced in vivo. The resistance of these microbes is comparable to, or slightly greater than, that demonstrated for other microorganisms, in particular Legionella pneumophilia, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus, and Mycobacterium tuberculosis (7,17,20,30). Production of either ·OH by addition of 10 mM Fe to H_2O_2 or ·I by the addition of Fe+I to H_2O_2 inhibits growth of M. hominis and U. urealyticum at approximately one log mM concentration less of H_2O_2 compared to H_2O_2 alone. In particular, U. urealyticum is slightly more sensitive to H_2O_2+Fe+I, whereas M. hominis is slightly more sensitive to H_2O_2+Fe.

Some authors have demonstrated convincing evidence that microorganisms that are catalase deficient are more susceptible to oxidant injury than microorganisms with adequate levels of this enzyme (1,27). Other investigators have demonstrated little relationship between catalase levels and

resistance to H_2O_2 (30). To test the importance of catalase inhibition in mycoplasmas, we used aminotriazole, a potent irreversible inhibitor of metaloproteins such as myeloperoxidase and catalase, to test the sensitivity of U. urealyticum and M. hominis to H_2O_2 when catalase is inhibited (5).

Aminotriazole, by itself, did not affect the growth of either U. urealyticum or M. hominis. U. urealyticum incubated with aminotriazole could tolerate approximately one greater log mM concentration of H_2O_2 compared to untreated microorganisms. This data suggest that catalase is not needed by U. urealyticum for protection from H_2O_2, $\cdot OH$, or $\cdot I$ and that the mechanism of protection afforded by aminotriazole could be inhibition of a metaloprotein, possibly catalase. Catalase has been shown to catalyze the reaction needed to produce a hypohalide, in much the same way that myeloperoxidase catalyzes this reaction (11). Although speculative, inhibition of catalase or other metaloprotein, thus preventing the production of a hypohalide, could explain the increased resistance to H_2O_2. Arguing against this speculation is that U. urealyticum has never been shown to contain catalase.

Conversely, M. hominis is more susceptible to oxidant injury after it has been pretreated with aminotriazole. This inhibitory effect by aminotriazole implies that catalase is important in protecting M. hominis from oxidant injury. This inhibitory effect is only seen in the presence of H_2O_2+Fe or H_2O_2+Fe+I suggesting that production of $\cdot OH$, either alone or in the presence of a halide, is responsible for the increased susceptibility to H_2O_2. Thus, catalase is

responsible for protecting M. hominis from oxidant injury, probably by preventing the production of the ·OH.

Although H_2O_2 can inhibit the growth of microorganisms, ·OH appears to be much more potent in promoting injury to microbes. Klebanoff et al. demonstrated the importance of ·OH in the killing of microorganisms, either directly or as an intermediate in the production of a hypohalide (16). Data from our experiments demonstrate the importance of ·OH in the killing of mycoplasmas.

The H_2O_2+Fe+I system is used as a model to test the sensitivity of microorganisms to oxidant-mediated killing by phagocytes (17). Our data suggest that phagocytes with intact oxidant-mediated killing mechanisms should be able to effectively kill these mycoplasmas. Conversely, phagocytes which possess defective oxidant-mediated killing would have to rely on other mechanisms to kill these microorganisms. Host organisms with multiple deficiencies in their immune system may not be able to eradicate these microorganisms at all. For example, U. urealyticum has been shown to persist in patients with hypogammaglobulinemia (25). Interestingly, the tissues usually affected are those with very low oxygen tensions, such as bone and joints (12,29). These tissues may be more susceptible in these patients because the phagocytic respiratory burst may be impaired due to the relatively low oxygen tensions (24).

These data have important implications in neonatal lung disease. Immature neonates have phagocytes that may not function well even in

normoxic environments (23). Unfortunately, these infants often require supplemental oxygen because of their pulmonary immaturity, underlying pulmonary disease, or both (28). Over time, high concentrations of oxygen decrease the respiratory burst in both neutrophils and alveolar macrophages, thus rendering them inefficient phagocytes (24). The immature neonate then becomes similar to the person who has hypogammaglobulinemia and osteomyelitis; they have a deficient immune system and their phagocytes cannot function well because of the local environment. This would explain why we were able to demonstrate the persistence of U. urealyticum in the tracheas of certain neonates over many months time. Since M. hominis may be able to produce H_2O_2, as many mycoplasmas can, they would provide the necessary substrate in the phagolysosome to promote their own killing and a phagocytic respiratory burst would not be needed (22). This could explain why we have not been able to demonstrate the persistence of M. hominis in the lower respiratory tracts of very immature neonates. These speculations need to be questioned and answered systematically because of their importance in the understanding of neonatal pulmonary clearance.

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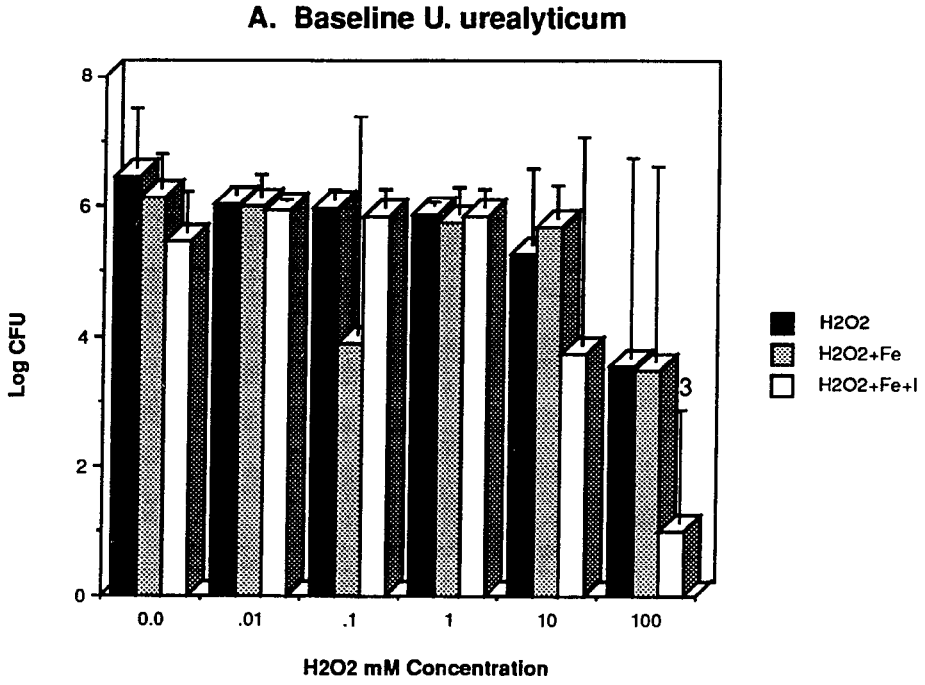
Table 1. Comparison of U. urealyticum and M. hominis Survival in Experimental Media[1]

Organism	Media[2]	Baseline	4 Hour	% Change[3]
U. urealyticum	10B-YE	6.5603	6.5267	0.5
	MEM	6.0603	6.0414	0.3
M. hominis	10B+YE	4.0207	4.0966	1.9
	MEM	4.0412	4.0837	1.1

1. Data are log transformed
2. 10B medium without yeast extract (10B-YE), 10B medium with yeast extract (10B+YE), Minimal essential medium (MEM)
3. Percent change from baseline

Figure 1. Effect of varying concentrations of H_2O_2, H_2O_2+Fe, and H_2O_2+Fe+I on growth inhibition of U. urealyticum. A. Samples obtained at time 0. B. Samples obtained after 4 hours of incubation at 37°C. The bars and error bars represent the mean and standard deviation of three experiments. Numerals represent significant differences from the corresponding control (0.0 mM H_2O_2): 1 = $p<0.005$, 2 = $p<0.01$, and 3 = $p<0.05$. Letters represent significant differences between the baseline and 4 hour samples: a = 0.005, b = 0.01, c = 0.05.


A. Baseline U. urealyticum
Log CFU
8
6
4
2
0
0.0
.01
.1
1
10
100
H2O2 mM Concentration
H2O2
H2O2+Fe
H2O2+Fe+I

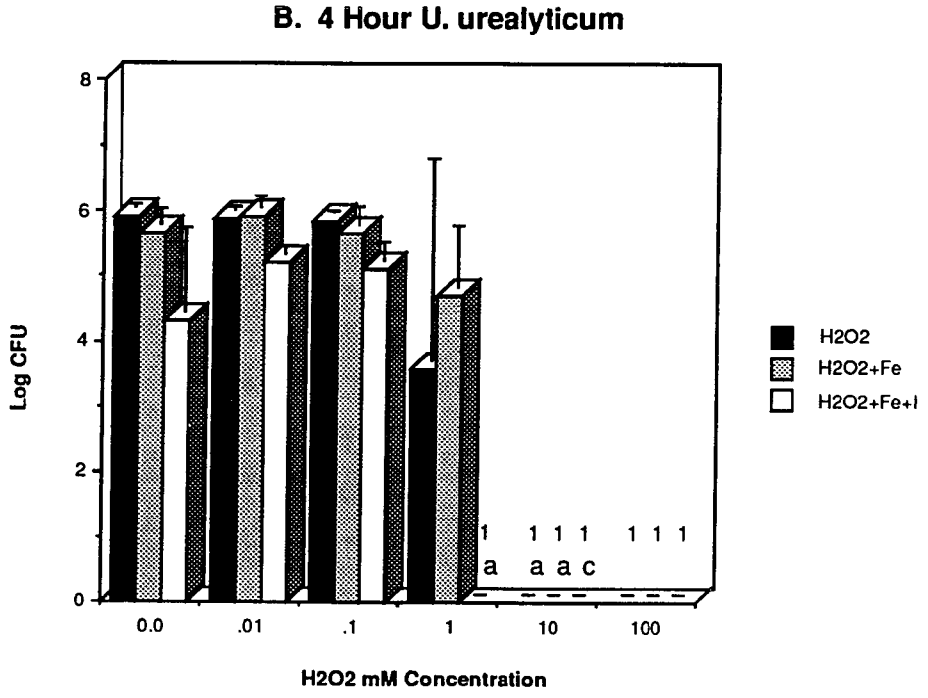
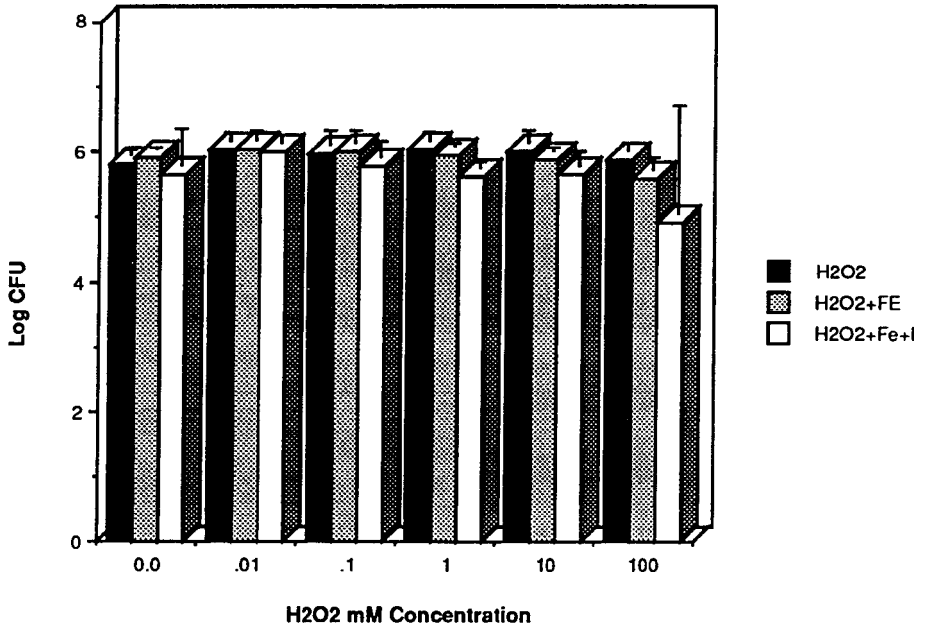

B. 4 Hour U. urealyticum
Log CFU
8
6
4
2
0
1 1 1 1 1 1 1
a a a c
0.0
.01
.1
1
10
100
H2O2 mM Concentration
H2O2
H2O2+Fe
H2O2+Fe+I

Figure 2. Effect of varying concentrations of H_2O_2, H_2O_2+Fe, and H_2O_2+Fe+I on growth inhibition of U. urealyticum, which have been incubated in 10 mM aminotriazole. A. Samples obtained at time 0. B. Samples obtained after 4 hours of incubation at 37°C. The bars and error bars represent the mean and standard deviation of three experiments. Numerals represent significant differences from the corresponding control (0.0 mM H_2O_2): 1 = $p<0.005$, 2 = $p<0.01$, and 3 = $p<0.05$. Letters represent significant differences between the baseline and 4 hour samples: a = 0.005, b = 0.01, c = 0.05.

A. Baseline U. urealyticum With Aminotriazole



B. 4 Hour U. urealyticum With Aminotriazole

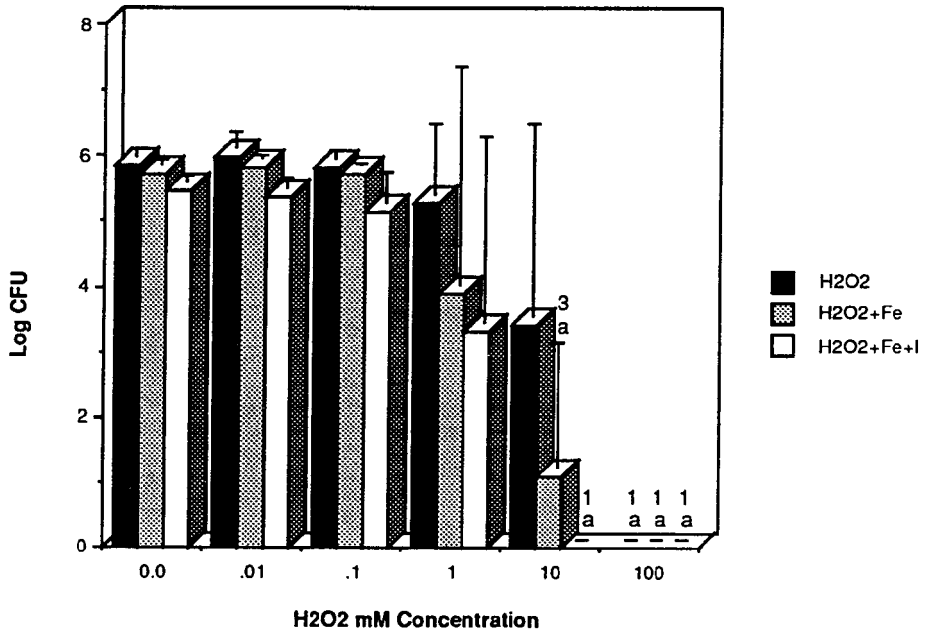
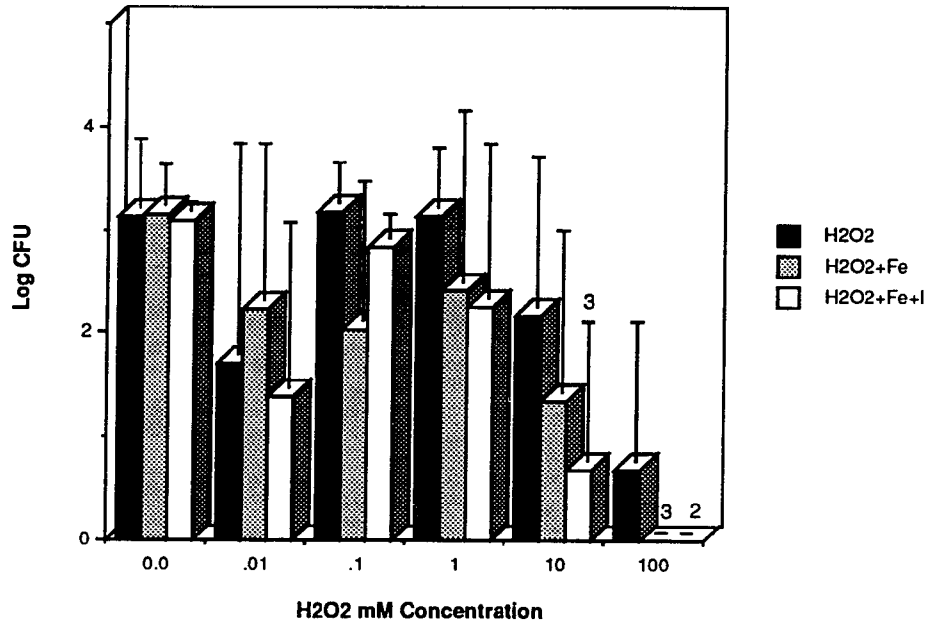


Figure 3. Effect of varying concentrations of H_2O_2, H_2O_2+Fe, and H_2O_2+Fe+I on growth inhibition of M. hominis. A. Samples obtained at time 0. B. Samples obtained after 4 hours of incubation at 37°C. The bars and error bars represent the mean and standard deviation of four experiments. Notice that the variability as shown by the standard deviation tends to be greater than with U. urealyticum. Numerals represent significant differences from the corresponding blank (0.0 mM H_2O_2): 1 = $p<0.005$, 2 = $p<0.01$, and 3 = $p<0.05$. Letters represent significant differences between the baseline and 4 hour samples: a = 0.005, b = 0.01, c = 0.05.

A. Baseline M. hominis



B. 4 Hour M. hominis

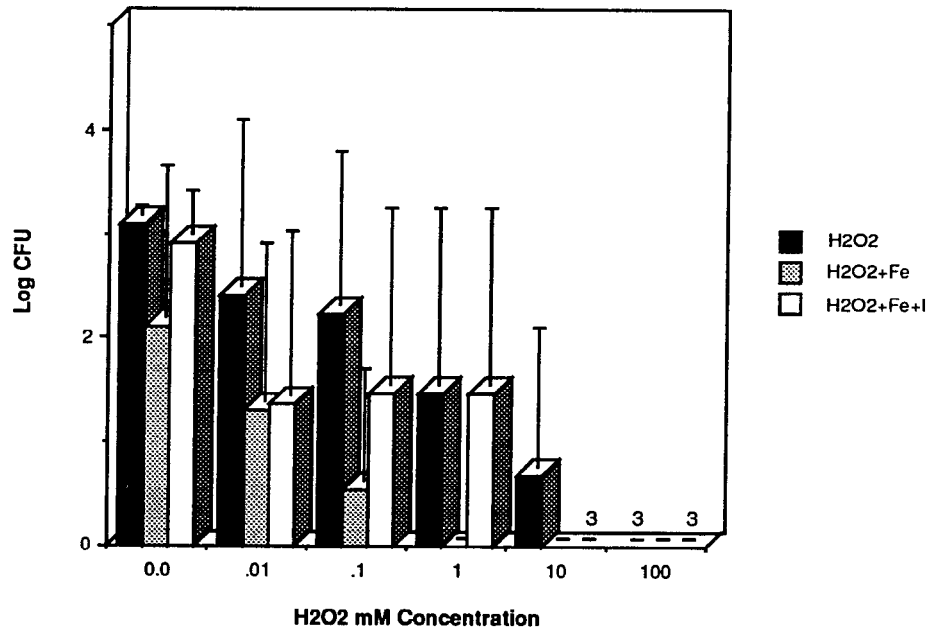
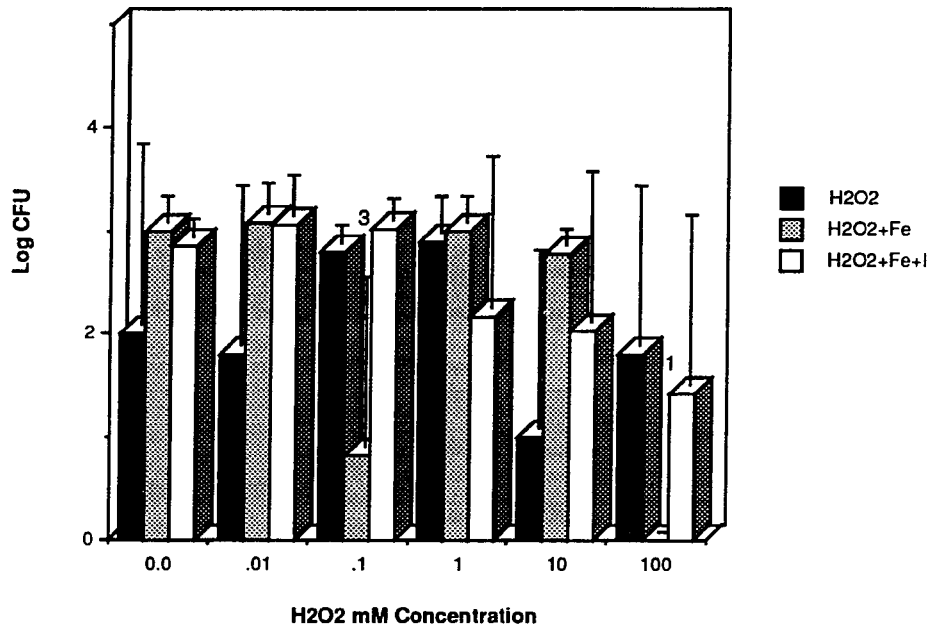
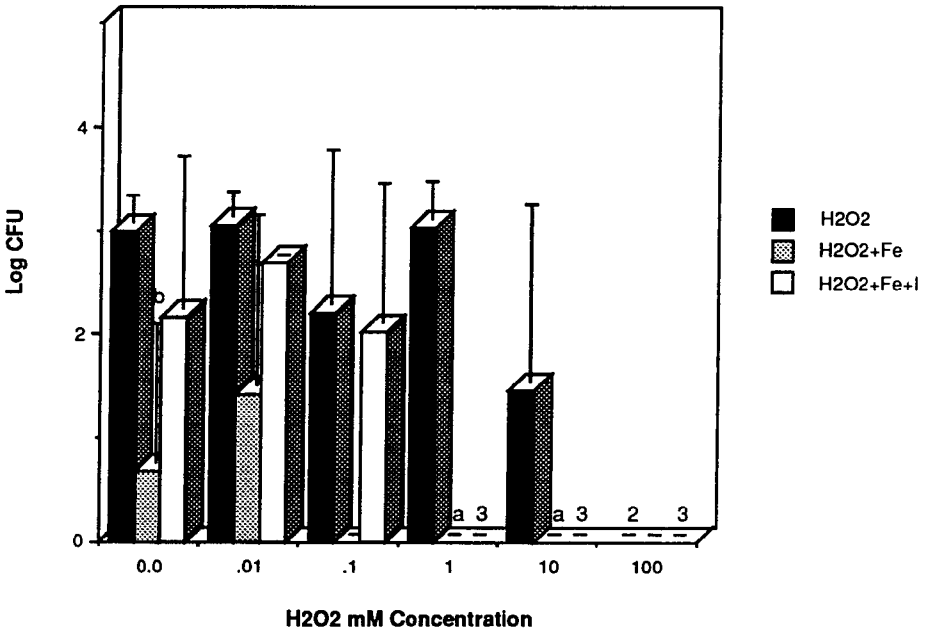


Figure 4. Effect of varying concentrations of H_2O_2, H_2O_2+Fe, and H_2O_2+Fe+I on growth inhibition of M. hominis which have been incubated in 10 mM aminotriazole. A. Samples obtained at time 0. B. Samples obtained after 4 hours of incubation at 37°C. The bars and error bars represent the mean and standard deviation of four experiments. Again, the variation between samples tends to be greater than with U. urealyticum. Numerals represent significant differences from the corresponding blank (0.0 mM H_2O_2): 1 = $p<0.005$, 2 = $p<0.01$, and 3 = $p<0.05$. Letters represent significant differences between the baseline and 4 hour samples: a = 0.005, b = 0.01, c = 0.05.

A. Baseline M. hominis With Aminotriazole



B. 4 Hour M. hominis With Aminotriazole



SUMMARY AND CONCLUSIONS

Previous reports have demonstrated that U. urealyticum is a cause of neonatal pneumonia, which can be fatal, and that tracheal or nasopharyngeal isolation of this organism is associated with chronic lung disease, death, or both in very premature infants with acute respiratory disease (16,18,60,92,105,142,145). The incidence and significance of ureaplasmal pneumonia in the human neonate are not completely defined. Whether tracheal isolation indicates infection of the respiratory tract, colonization of the conducting airways, or both, is speculative. Walsh et al. performed open lung biopsies on eight infants with chronic lung disease and isolated U. urealyticum from four of the tissue specimens (144). Particularly interesting was that U. urealyticum was only isolated from two of the four infants. This raises some doubt as to the sensitivity of the tracheal aspirate to identify this organism in the lower respiratory tract. Given the lack of knowledge concerning the occurrence and progression of ureaplasmal disease in human neonates, we embarked on a study to examine abnormal changes on chest radiographs of premature infants with respiratory disease and to associate these changes with the isolation of microorganisms from the trachea.

We argued that if U. urealyticum causes respiratory infection, not just colonization, in a large number of infants, then radiographic changes on chest x-rays taken routinely during the hospital course ought to reflect this process.

I recognized that premature infants with this organism isolated from their trachea seemed to have a more rapid progression of radiographic changes consistent with chronic lung disease than infant without this organism in their trachea. This progression was more consistent with the original radiographic description of bronchopulmonary dysplasia than the chronic lung disease seen today (84). First we hypothesized that U. urealyticum would be associated with pneumonic changes on chest radiographs. Our data support this hypothesis. This is significant because chest radiographs consistent with pneumonia rarely correlate with the isolation of a pathologic organism (1). These studies probably lacked statistical power because cultures for the most commonly isolated organism from the respiratory tract of premature infants with respiratory disease, U. urealyticum , were not obtained. Our data also support the clinical impression that infants with U. urealyticum have a more rapid progression of their respiratory disease. We termed this progression "precocious dysplastic changes." We speculated that if U. urealyticum causes pneumonia and chronic lung disease in human neonates, then these infants should be one and the same. Again our data supported our assumptions. Infants with pneumonic changes on chest radiograph were more likely to develop precocious dysplastic changes than those without pneumonic changes. Although infants with precocious dysplastic changes went on to develop chronic lung disease, a statistical difference could not be demonstrated between the number of infants with and without U. urealyticum who developed radiographic changes of chronic lung disease. This is likely due to the very broad definition of chronic lung disease that was used in the study. The definition used counted all

abnormal radiographs at 28 days of age as indicative of chronic lung disease, whereas many infants with radiographic evidence of chronic lung disease did not need supplemental oxygen. Thus, we demonstrated that tracheal isolation of U. urealyticum is associated with radiographic changes consistent with pneumonia and chronic lung disease. These data give additional credence to the theory that tracheal isolation is indicative of true infection in a large number of infants.

Neonates who later develop evidence of ureaplasmal lung disease seldom, if ever, have normal, undiseased lungs initially. The infants most often associated with U. urealyticum disease are premature and have acute lung disease requiring supplemental oxygen (18). Pneumonia caused by U. urealyticum may even start in utero, prior to birth (16,91). Also, infants receive respiratory, antimicrobial, and other therapies to support their bodily functions until they can manage on their own. Therefore, observing the normal course of ureaplasmal respiratory disease without confounding variables is impossible in humans. Therein lies the importance of the newborn mouse model of U. urealyticum pneumonia developed by Rudd et al. (102).

A common finding in all of the studies which associate U. urealyticum with chronic lung disease is that all of the infants studied had acute lung disease requiring supplemental oxygen (18,60,105,145). Oxidants, such as oxygen, nitrogen dioxide, and ozone, are extremely toxic to the respiratory system (23,24). Parker et al. has shown that nitrogen dioxide decreases the pulmonary clearance of Mycoplasma pulmonis by impairing intrapulmonary killing (88). Dunn et al. has shown that hyperoxia decreases the pulmonary

clearance of Pseudomonas aeruginosa by decreasing the influx of neutrophils into the lung (39). Hyperoxia also decreases the respiratory burst of phagocytic cells and impairs their oxygen-associated microbial killing (130).

We used the newborn mouse model of ureaplasmal pneumonia to study the impact of hyperoxia on the pneumonic process. Newborn mice inoculated intranasally with broth containing U. urealyticum and then maintained in 80% oxygen were severely affected. The organisms persisted for a much longer period of time in the mice exposed to hyperoxia, suggesting that pulmonary clearance was impaired. The pulmonary lesions also were worse in the hyperoxia exposed mice. The lesions consisted of increased alveolar macrophages, influx of rare neutrophils, alveolar thickening, proteinaceous debris, and occasional hemorrhage. Interesting to note is the relative absence of neutrophils in the pulmonary lesions of mice exposed to hyperoxia. A similar finding was made in mice inoculated with Pseudomonas aeruginosa and placed in a hyperoxic environment by Dunn et al. (39). A striking feature of these experiments was that the mortality paralleled the peak of the pulmonary lesions. Mortality was only seen in mice exposed to hyperoxia. This experiment supports our assumption that hyperoxic environments potentiate ureaplasmal persistence and pneumonia in newborn mice, and that similar relationships probably occur in the human neonate.

Older mice are relatively resistant to U. urealyticum pneumonia. Adult and juvenile (12-14 day old) mice were exposed to hyperoxia for 24-48 hours to determine if hyperoxia increased the susceptibility of immunologically mature mice to this microbe. Hyperoxia increased organism persistence and lesion

severity, but none of the animals died. Again, an unusual feature was the occurrence of hemorrhage in some of the mice exposed to hyperoxia and inoculated with U. urealyticum. Although postmortem extravasation of blood from the engourged pulmonary vessels in the mature mice could have occurred to produce what appeared to be focal hemorrhage. These data confirm that pneumonia can be produced, even in mice that are resistant to U. urealyticum, if they are pre-exposed to elevated concentrations of oxygen. Oxygen can exacerbate pneumonic processes due to this agent in both immature and mature mice, an effect that may be operative in human patients.

U. urealyticum persisted longer in the respiratory tracts of mice exposed to oxygen in both experiments conducted here. In addition, Cassell et al. demonstrated that these microbes could persist for almost 6 months in the respiratory tracts of infants on supplemental oxygen (18). Because this organism would have been exposed to toxic oxygen species from several sources within the affected lungs and survived, we exposed this microbe to toxic oxygen species in vitro. Toxic oxygen species are produced by phagocytic cells in inflammatory infiltrates (5). Hyperoxia also increases the production of these species by normal pulmonary parenchymal cells (45) Also, U. urealyticum might produce oxygen radicals as part of normal metabolism as do several other mycoplasmas (57). We speculated that since U. urealyticum persisted in an environment where toxic oxygen species were likely to exist, that these microbes must be rather resistant to oxidant injury. Our data supported these assumptions. U. urealyticum is killed only at very high levels of H_2O_2, H_2O_2 +

Fe, and H_2O_2 + Fe + I. Thus, these organisms should be able to persist in a rather unfriendly atmosphere with some ease.

Two properties of U. urealyticum might make them more resistant to intrapulmonary killing. These microbes colonize ciliated epithelium. No inflammatory infiltrates in the airways were detected in any of our experiments. Therefore, the level of oxygen radicals in these airways would presumably be low. Also, U. urealyticum might be able to survive inside of phagocytes (149). Again, this would protect them from the surrounding environment. These mechanisms of organism persistence need to be examined because of the importance of this possible reservoir in the human neonate.

Future Strategies Current evidence implicates U. urealyticum as a common neonatal pathogen. Our data confirm the ability of this organism to cause disease in mice, thus fulfilling Koch's postulates, and the capability of hyperoxia to potentiate ureaplasmal respiratory disease. Proving causation, however, may be difficult. A treatment trial to prevent chronic lung disease by an appropriate antibiotic is the most logical next step in evaluating human disease. If the antibiotic decreases chronic lung disease in infants with U. urealyticum, the issue of causality could be put to rest. This may be hopeful thinking. The most appropriate antibiotic is erythromycin because of its relative lack of toxicity (143). This antibiotic is only bacteriostatic and may not eradicate these organisms from the respiratory tract with great efficiency (52). Thus, the pulmonary inflammation may persist, continuing the pulmonary injury. Even if this antibiotic is effective, infants who might have died from respiratory disease

might survive with chronic lung disease, thus effectively increasing the number of infants with chronic lung disease. Therefore, a treatment trial should not be undertaken without understanding the possibilities of a negative effect and how to interpret such results.

The newborn and adult mouse model can be used to test the effects of altering the pulmonary inflammatory reaction. Two substances which human infants receive that might do this are surfactant and steroids (4,25,119). The effects of these modalities on pulmonary infection are incompletely understood. Also, the effects of age on pulmonary disease can be studied, as can the effects of therapies on pulmonary clearance. Understanding the pneumonic process produced in mice and the effects of our current therapies and newer modalities should help guide the selection and use of future therapies in human neonates.

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