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ARTICLES

Effects of Antenatal Colonization with *Ureaplasma urealyticum* on Pulmonary Disease in the Immature Baboon

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ABSTRACT

Current nonhuman models for bronchopulmonary dysplasia have not included perinatal infection. We studied the effects of antenatal *Ureaplasma urealyticum* (*Uu*) infection in the 125-d immature baboon. Ten 125-d gestation (term = 185 d) baboon dams were delivered after intra-amniotic inoculation with *Uu*. Serial blood and tracheal aspirate samples were analyzed for *Uu* colony-forming units, IL-6, IL-8, and cell counts. Physiologic parameters were serially recorded. Lung histology was examined after 14 d of ventilation and compared with unexposed controls. All *Uu*-exposed animals had $>4 \times 10^2$ CFU in tracheal aspirate at 24 h. Four of nine *Uu* animals remained heavily colonized [(+) *Uu*] at necropsy ($>6 \times 10^3$). Five animals had negative or low tracheal colony-forming units. All *Uu* animals had significant increases for white blood cells, IL-6, and IL-8 in amniotic and fetal lung fluid. Compared with controls, (+) *Uu* animals had significantly higher fraction of inspired oxygen, airway pressures, oxygenation index, and ventilation efficiency index between 48 and 240 h and had significantly elevated tracheal IL-6 and IL-8 concentrations between 72 and 240 h. Compared with controls (−) *Uu* animals had significantly better oxygenation index and ventilation efficiency index scores between 48 and 144 h. Lung histopathology in both *Uu* groups showed more severe bronchiolitis and interstitial pneumonitis compared with controls. Two patterns of disease were observed after *Uu* perinatal infection. Persistent colonization manifested a picture consistent with acute pneumonitis, worse lung function from 2 to 10 d, and prolonged elevated tracheal cytokines. Colonized animals that subsequently cleared *Uu* from the lung demonstrated early improved lung function compared with unexposed controls yet still manifested mixed bronchiolitis and interstitial pneumonitis at necropsy. Inherent immune system responses may determine outcome of perinatal *Ureaplasma* colonization. **(*Pediatr Res* 54: 797–807, 2003)**

Abbreviations

CFU, colony forming unit
***Uu*,** *Ureaplasma urealyticum*
BPD, bronchopulmonary dysplasia
TA, tracheal aspirate
OI, oxygenation index
VEI, ventilatory efficiency index
Fio_2, fraction of inspired oxygen
$Paco_2$, arterial blood partial pressure carbon dioxide
Pao_2, arterial blood partial pressure oxygen
Paw, mean airway pressure

The pathogenesis of bronchopulmonary dysplasia (BPD) is incompletely understood, but pulmonary inflammation seems to play a critical key role (1–3). Pulmonary inflammation may be initiated by various insults, including hyperoxia, volutrauma, and infection. Recent reports suggest an important role for both postnatal and perinatal infection in the pathogenesis of BPD (4–7).

Evidence for microbial invasion of the amniotic cavity has been found in up to 80% of preterm births (8). The organism most commonly identified has been *Ureaplasma urealyticum*

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(*Uu*)(9–11). The presence of *Uu* in the preterm respiratory tract has been correlated with elevated cellular and molecular markers of inflammation and associated with an increased risk for BPD (7, 12–14).

Current nonhuman models for BPD are limited by the lack of perinatal infection as a confounding factor. A previous pilot study from this center demonstrated worse clinical and histologic hyaline membrane disease after *Uu* colonization of the 140-d preterm baboon (15). However, *Ureaplasma* colonization was conferred postnatally, and all animals were exposed to hyperoxia [fraction of inspired oxygen (Fio_2) 1.0]. Our current BPD model involves the more immature 125-d baboon, which manifests clinical and pathologic features similar to immature humans at risk for BPD today (16). These animals develop BPD in the presence of antenatal steroid therapy and prophylactic surfactant replacement therapy coupled with low-tidal-volume mechanical ventilation and appropriate oxygen support. The objective of this study was to characterize the clinical and pathologic response of the 125-d immature baboon to intrauterine amniotic fluid colonization with *Uu*.

METHODS

***Introduction of intra-amniotic* Uu.** All animal studies were performed at the Southwest Foundation for Biomedical Research in San Antonio, Texas. All animal husbandry, animal handling, and procedures were reviewed and approved by the Institutional Animal Care and Use Committee and conformed to American Association for Accreditation of Laboratory Animal Care guidelines. At 122–123 d timed gestation, 10 pregnant baboons (*Papio papio*) were anesthetized with ketamine hydrochloride (10 mg/kg, i.m.) and restrained in a supine position. Under ultrasound guidance, an 18-gauge needle was inserted through aseptically prepared skin into the amniotic cavity and 10 mL of amniotic fluid was withdrawn. Five milliliters of fluid was allocated for microbial cultures (bacterial and *Ureaplasma*), cell count and differential, and cytokine assays as described below. The remaining 5 mL of sterile amniotic fluid was mixed with 5 mL of *Uu*, serovar subtype 1, suspended in 10B broth at a concentration of 10^7 colony-forming units (CFU), and infused back into the amniotic cavity. Dams were recovered in normal manner and quarantined for 48–72 h before elective cesarean delivery under general anesthesia at 125 ± 2 d (term = 185 d). All dams were treated postoperatively with azithromycin (Eli Lilly, Indianapolis, IN, U.S.A.) for 7 d. Animals were not introduced back into the active colony until vaginal cultures and PCR were negative on two consecutive specimens. Neither *Ureaplasma*-exposed nor control animals received antenatal steroids before delivery. This was a pilot study to determine the feasibility and effects of antepartum *Ureaplasma* exposure in our immature animals model for BPD. The control animals also served as controls to more established projects within our BPD research program, which did not allow for sham intrauterine amniocentesis at the designated time.

Delivery and instrumentation. At birth, infants were weighed, sedated (ketamine HCl, 10 mg/kg), and intubated with a 2.5-mm endotracheal tube. Before the first breath, tracheal lung liquid was collected, then infants received a single bolus (100 mg/kg) of exogenous surfactant (Survanta, donated by Ross Laboratories, Columbus, OH, U.S.A.). Ventilation was initiated in all infants with a pressure-limited, time-cycled infant ventilator (InfantStar, donated by Infrasonics, San Diego, CA, U.S.A.) as previously described (16, 17). Animals were instrumented with an umbilical arterial catheter and percutaneous central venous catheter and nursed in a servocontrolled, infrared-warmed body plethysmograph (VT1000, VitalTrends Technology, New York, NY, U.S.A.). Intermittent sedation was provided as needed with ketamine (5 mg/kg) and/or diazepam (0.1 mg/kg).

Ventilator management and pulmonary function testing. The ventilatory approach applied has been previously described (16, 17) Briefly, pressures were adjusted to maintain tidal volumes at 4–6 mL/kg as measured by plethysmography and associated with adequate chest motion by clinical examination. Rate was adjusted to regulate arterial blood partial pressure carbon dioxide ($Paco_2$) between 45 and 55 torr. Target goals for Pao_2 were 55–70 torr. Oxygenation was primarily manipulated through changes in positive end expiratory pressure and Fio_2. Oxygenation index [OI; Fio_2*100*mean airway pressure (Paw)/arterial blood partial pressure oxygen (Pao_2)] and ventilation efficiency index (VEI; $Paco_2$ * rate * peak inspiratory pressure/1000) were used as indices for oxygenation and ventilation. Pulmonary function testing was performed using the VT1000 body plethysmograph as previously reported (16, 17). Briefly, 10 breaths, meeting predefined breath selection criteria, were recorded at each time point and averaged for determination of tidal volume (mL/kg), dynamic respiratory system compliance ($mL \cdot cm\ H_2O^{-1} \cdot kg^{-1}$), and expiratory resistance ($cm\ H_2O \cdot L^{-1} \cdot s^{-1}$). Variability between measurements was compared by periodic triplicate recording of pulmonary function tests and was consistently <10%.

Other care plans. Animals were not enterally fed during this 14-d study. Parenteral nutrition was initiated at 24 h of life (16, 17). Arterial blood gases, hematocrit, and serum chemistries were measured at designated time intervals. Hematocrit was maintained between 30 and 45% through periodic administration of fresh, heparinized packed red cells obtained from adult baboons. All animals were treated with antibiotics (ampicillin and amikacin) for the duration of the study. Prophylactic fluconazole was initiated in all animals with $6.0\ mg \cdot kg^{-1} \cdot dose^{-1}$ at 12 h of age and repeated at 96, 168, and 240 h of age.

Microbiology. The following specimens were processed for culture and PCR for *Uu*: amniotic fluid, fetal lung liquid, endotracheal aspirate, fetal membrane, decidua, periodic blood samples, lung, and brain. Specimens were transported in 10B media for culture and PCR transport buffer. Amniotic fluid was concentrated before inoculating the transport media. Fluid was centrifuged for 10 min at 10,000 rpm, and the supernatant was removed. The pellet was resuspended in 1 mL of residual fluid, and 300 μL was added to the transport media. Samples (150 μL) of fetal lung liquid and endotracheal aspirates, collected as described below, were added to each of the transport media. Fetal membrane cultures were collected by swabbing between the chorion and the amnion with three Dacron swabs. Swab 1 was inoculated into culture media, swab 2 was inoculated into

PCR buffer, and swab 3 was inoculated into sucrose phosphate buffer for storage. Swabs were rinsed in the respective vials and swirled, excess fluid was removed by pressing the swab against the inside of the vial, and the swab was discarded. At necropsy, all tissue samples were finely minced with scissors and sterile blades in 2 mL of sterile sucrose phosphate buffer. A 300 μL sample of each tissue specimen was added to a culture transport and a PCR buffer. Specimens were frozen immediately upon collection at −70°C until transported on dry ice to the Diagnostic Mycoplasma Laboratory at the University of Alabama Birmingham. Once received at the Diagnostic Mycoplasma Laboratory, culture specimens were thawed and processed by quantitative culture using 10B broth and A8 agar for *Uu*. Previously published protocols were used (18). PCR for *Uu* was performed on batched specimens using standard techniques. The primers for *Uu* were directed toward the urease gene (19).

Pathology: light microscopy. At necropsy, the right lower lobe was removed, weighed, and intrabronchially fixed with phosphate-buffered 4% paraformaldehyde and 0.1% glutaraldehyde at 20 cm H_2O constant pressure for 24 h. After fixation, the volume of the right lower lobe was determined by volume displacement. The lobe was cut into three serial, equally spaced horizontal tissue sections. The entire cut surfaces of all three horizontal sections were processed for light microscopic study. These specimens were dehydrated in alcohol, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin. Airway inflammation and interstitial pneumonitis was subjectively assessed and graded as absent, mild, and moderate-severe, and the number of intra-alveolar macrophages were judged to be minimal or numerous in quantity.

Tracheal aspirates. Tracheal aspirates (TA) were collected at approximately 24, 72, 120, 240, and 336 h as previously described (16, 17). Each aspirate was separated for cell count and differential specimens (transferred by Nunc tube on wet ice to the laboratory); cytokine samples were centrifuged for 10 min at 2500 rpm, and the supernatant was removed and allocated in 0.25-mL aliquots, then frozen at −70°C until assay. IL-6 was measured using a specific antiserum to human IL-6 (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a final dilution of 1:100,000, with radiolabeled human IL-6 from New England Nuclear and purified human IL-6 for the standard (Austral Biologicals, San Ramon, CA, U.S.A.). Assay sensitivity was 0.6 pg/tube, and the intra- and interassay coefficients of variation were 6.5% and 11.9%, respectively. An enzyme immunoassay (Applied Biosystems, Foster City, CA, U.S.A.) was used to measure IL-8. Assay sensitivity was 100 pg/mL, and the intra-and interassay coefficients of variation were 10% and 24%.

Data analyses. Data are presented as mean ± SEM or median with interquartile ranges. Between-group differences for continuous data were compared by unpaired *t* tests, Mann-Whitney *U* test, ANOVA, or Kruskal-Wallis tests as indicated. Ordinal data were compared by Mann-Whitney *U* or Kruskal-Wallis tests. Categorical data were compared by Fisher's exact test or χ^2. Comparisons among various time points were made using one-way ANOVA for repeated measures. $P < 0.05$ was required for significance. Statistical results were generated using SPSS, version 9.0 (SPSS, Chicago, IL, U.S.A.).

RESULTS

None of the dams was culture or PCR positive for *Uu* in the amniotic fluid before inoculation, but all had positive amniotic fluid cultures at delivery (range 3.60×10^4 to 2.05×10^7). This range of CFUs is consistent with those found in a group of human women delivered by cesarean section at <34 wk gestation with histologic chorioamnionitis (Cassell G, Duffy L, unpublished data). None of the dams manifested signs of localized or systemic infection during the interval from inoculation to delivery, and there was no evidence for premature labor. Among dams inoculated with *Uu*, there was a marked rise in amniotic fluid levels for white blood cells, IL-6, and IL-8 at the time of delivery (Table 1), but there was no effect on maternal or neonatal serum levels for these variables. All placental tissues except one were inadvertently sent only for culture studies. In the single placenta available for microscopic examination after intrauterine *Uu* exposure, there was histologic evidence of acute inflammation of the chorioamniotic membranes, a finding not seen in any control placentas examined to date.

All fetuses exposed to intra-amniotic *Uu* were born alive. One infant died at 28 h of age as a result of severe cardiovascular compromise (data not included); all others survived until necropsy at 14 d of age. There were no differences between control infants and *Uu*-exposed infants for gestational age (125 ± 1 *versus* 125 ± 1 d), birth weight (408 ± 49 *versus* 391 ± 28 g), or sex (63% male *versus* 60% male). As shown in Table 2, serial TA cultures revealed a dichotomous pattern for *Uu*-exposed infants. Two discrete groups of infants were identified on the basis of the presence of high (+*Uu*; $n = 4$) *versus* low/no (−*Uu*; $n = 5$) *Uu* CFUs at necropsy. The median

Table 1. *Effect of intra-amniotic Uu colonization on amniotic fluid cytokine and white blood cell concentrations of baboon dams*

	Preinoculation ($n = 10$)	Delivery ($n = 10$)	*p*
IL-6*	1840 (1409–2417)	3242 (2741–5582)	0.016
IL-8*	311 (215–619)	10 214 (4617–43,690)	<0.001
Total white blood count †	428 (16–844)	15 000 (3500–110,249)	0.018
Neutrophil count†	32 (0–580)	13 125 (1407–100,688)	0.033
Macrophage/monocyte count †	0 (0–560)	1 500 (437–7500)	0.029

All values as median (25%–75%)
* pcg/mL.
† Cells/mL.

Table 2. *CFUs for Uu in amniotic fluid and TA of 125-day premature baboons*

Animal no.	AF pre-*Uu*	Incubation hours	AF delivery	TA 24 h	TA 72 h	TA 144 h	TA, necropsy
3F	0	48	4.4×10^5	7.5×10^3	6.0×10^5	2.1×10^5	6.2×10^3
3Q	0	48	1.0×10^6	9.0×10^3	1.2×10^5	2.0×10^2	4.5×10^4
4D	0	72	2.1×10^6	1.5×10^3	3.7×10^5	1.3×10^7	1.6×10^3
2F	0	72	9.5×10^5	1.1×10^5	2.1×10^6	4.1×10^6	2.2×10^5
3G	0	48	2.4×10^5	4.5×10^2	50	1	150
3Y	0	72	1.2×10^7	2.9×10^3	3.0×10^3	0	0
3X	0	48	5.2×10^6	3.7×10^4	3.7×10^6	1.0×10^2	0
1Y	0	72	6.4×10^4	2.5×10^5	5.6×10^4	7.9×10^3	0
2S	0	72	2.0×10^7	1.0×10^2	1	0	0

AF, amniotic fluid.

values for *Uu* CFUs from TAs were significantly different ($p < 0.05$, Mann-Whitney *U*) between these two groups by day 6 of life until necropsy.

Comparison between all *Uu*-exposed infants and controls revealed no differences in clinical, physiologic, or ventilatory variables at any designated interval from birth until necropsy (data not shown). However, comparisons between control infants and the *Uu* subgroups, (+) *Uu* and (−) *Uu* revealed important differences in lung function and histopathology.

Oxygenation was similar in all three groups until 48 h of age. From 48 h of age until 240 h (+) *Uu* infants required higher Fio_2 support at multiple time points compared with control and (−) *Uu* infants to achieve target Pao_2 (Fig. 1*A*) The need for higher Fio_2 among (+) *Uu* infants was accompanied by higher mean airway pressure (Fig. 1*B*). The end result of increased Fio_2 and mean airway pressure requirements in (+) *Uu* animals was a significant increase in OI from 48 h until 240 h as shown in Figure 1*C*. It is interesting that by necropsy at day 14, there were no differences in oxygenation parameters between (+) *Uu* animals and controls. There were divergent patterns for Fio_2, mean airway pressure, and OI between (+) *Uu* and (−) *Uu* animals. Whereas (+) *Uu* animals manifested worse oxygenation compared with controls, (−) *Uu* animals trended toward improved oxygenation compared with controls, with differences being significant at 5, 6, and 14 d (Fig. 1).

Measures of ventilation followed a course similar to oxygenation. There were no differences between groups until after 48 h of age. From 72 h age through 240 h age, the (+) *Uu* animals required increased peak inspiratory pressures (Fig. 2*A*) and increased ventilator rates (Fig. 2*B*) compared with control and (−) *Uu* animals. The VEI, a measure of the amount of ventilator support required to achieve a specific level of ventilation, is shown in Figure 2*C*. The VEI was significantly elevated from 48 through 240 h in (+) *Uu* animals compared with controls and (−) *Uu* animals and remained elevated at 336 h compared with (−) *Uu* animals. As with oxygenation, there were no significant differences for ventilation indices between controls and (+) *Uu* animals at the time of necropsy, and ventilation was more easily achieved in (−) *Uu* animals than in controls with significant differences found on days 5, 6, and 14 (Fig. 2).

Pulmonary function testing did not tend to mirror group differences found for oxygenation and ventilation parameters (Fig. 3). Initial (6 h of age) airway resistance was significantly higher among (+) *Uu* animals compared with controls and (−) *Uu* infants, but there was wide variability within and overlap between groups at the various time points compared (Fig. 3*A*). There was a consistent and significant decrease over time in airway resistance within each of the three study groups ($p < 0.05$ by repeated measures ANOVA). Dynamic respiratory compliance did not differ between groups over the initial several days of life (Fig. 3*B*). By day 10, there was a significantly lower compliance among (+) *Uu* animals compared with controls and (−) *Uu* animals, but at necropsy on day 14, this was reversed and control animals had a lower respiratory dynamic compliance than either of the *Uu* groups. Wide variability existed within groups, but over the 14-d study period, there was a significant improvement in compliance within each group ($p < 0.05$, repeated measures ANOVA).

We measured cytokine levels (IL-6 and IL-8 in blood and TAs) and white blood cell counts in TAs as indicators of systemic and pulmonary inflammation. There were no between-group differences at specified time points from birth to necropsy for concentrations of IL-6 or IL-8 in the serum (data not shown). There also were no differences within groups over time (repeated measures ANOVA) for serum cytokine levels. In contrast, IL-6 levels were dramatically higher in both *Uu* groups compared with controls in fetal lung liquid and 24-h TAs (Table 3). Although TA IL-6 concentrations decreased over time in all animals, they remained significantly higher in the (+) *Uu* animals compared with controls and (−) *Uu* animals from 72 h through 240 h. TA concentrations for IL-8 manifested a different pattern than IL-6 (Table 3). Among (−) *Uu* animals, IL-8 concentrations peaked at 24 h (significantly higher than controls) and then decreased to levels similar to controls for the remainder of the 14-d study period. IL-8 values increased from birth through 72 h for (+) *Uu* animals, then slowly decreased over time but remained significantly greater than control and (−) *Uu* animals until 240 h age. Although total white blood cells and percentage of neutrophils in TAs trended toward higher values in the (+) *Uu* animals, the only time point that was significantly different occurred at 72 h.

Histopathologic findings are shown in Table 4. Bronchiolitis, interstitial pneumonitis, and alveolar macrophage accumulations, when present, were focal in distribution in all animals. This focal involvement is important to note, because most of these lesions were microscopic, not macroscopic, and would likely not be discernible radiographically. Bronchiolitis was less commonly seen in the control group when compared with the *Uu* study animals (Figs. 4 and 5*A*). Interstitial pneumonitis

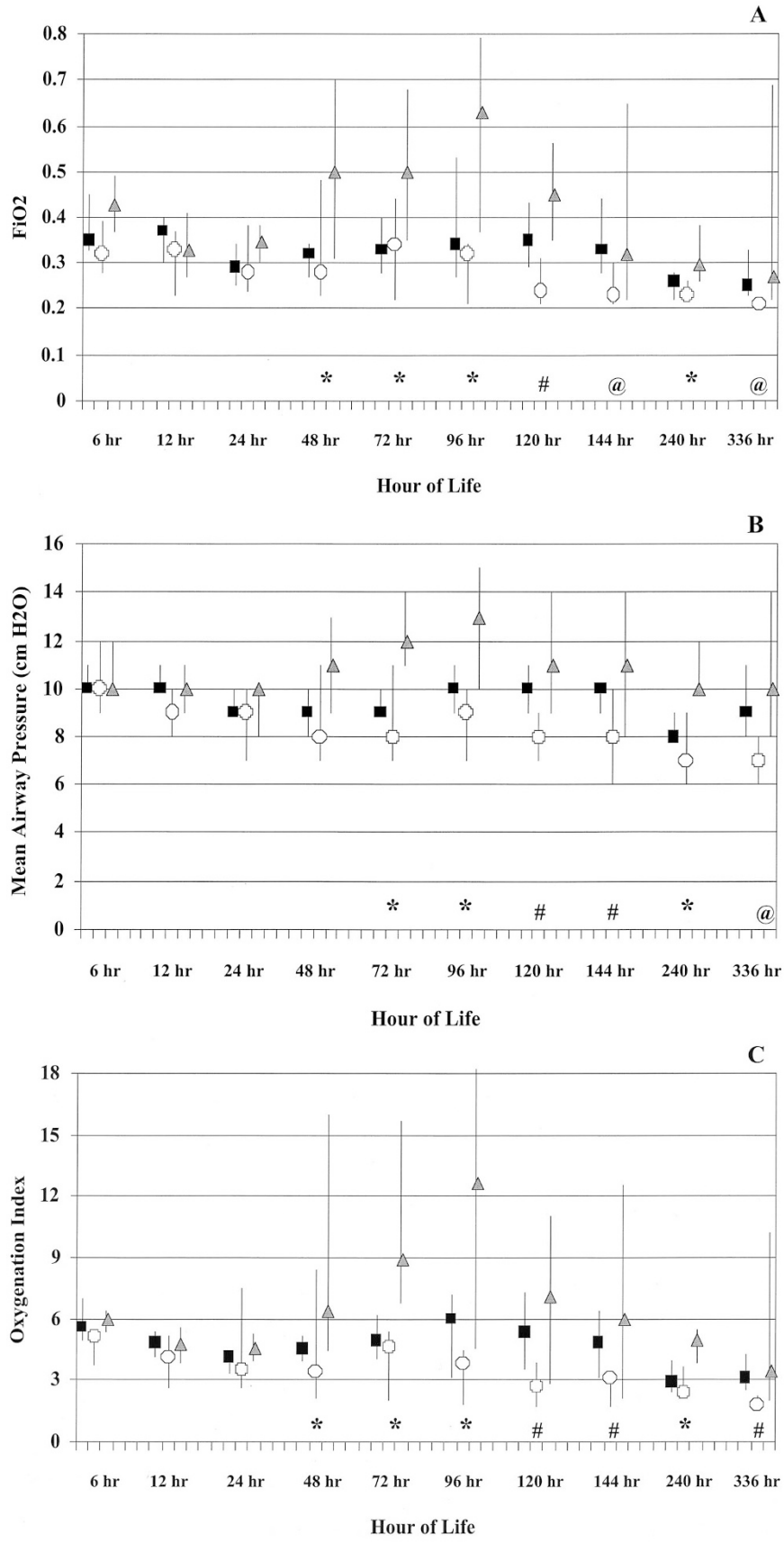

Figure 1. Effect of intra-amniotic *Uu* exposure on postnatal oxygenation in 125-d premature baboons. Data are expressed as median and quartiles. ■, unexposed controls; △, exposed infants that had persistently positive tracheal cultures [(+) *Uu*]; ○, exposed infants that subsequently cleared *Uu* from the trachea [(−) *Uu*]. (*A*) Fio_2. (*B*) Mean airway pressure (Paw). (*C*) OI (Fio_2 * 100 * Paw/Pao_2). *$p < 0.05$ for (+) *Uu vs* control and (−) *Uu*; #$p < 0.05$ for each group *vs* the others; @$p < 0.05$ for (−) *Uu vs* control and (+) *Uu*. ANOVA with Student-Newman-Keuls *post hoc* analysis.

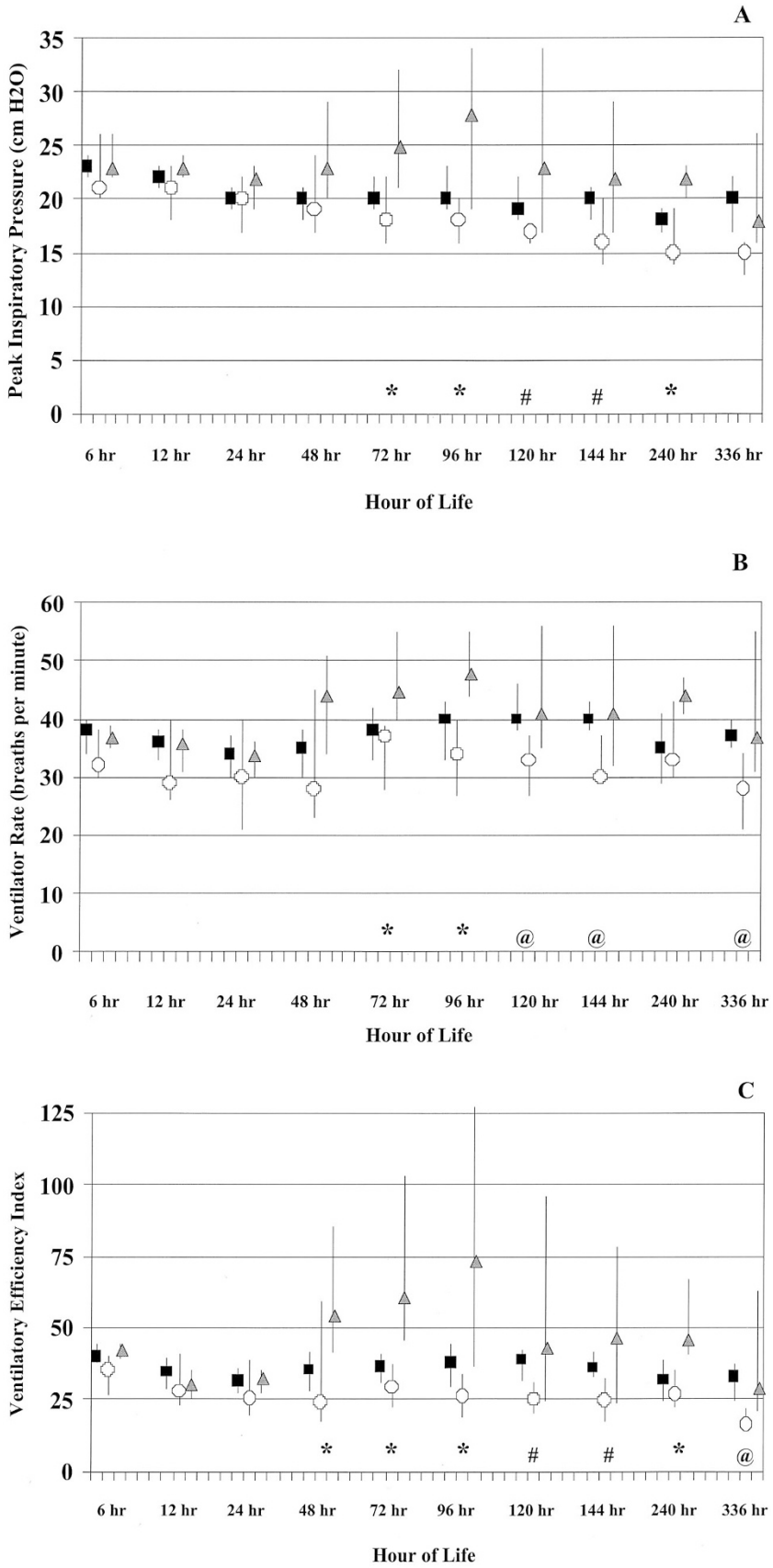

Figure 2. Effect of intra-amniotic *Uu* exposure on postnatal ventilation in 125-d premature baboons. Data are expressed as median and quartiles. ■, unexposed controls; △, exposed infants that had persistently positive tracheal cultures [(+) *Uu*]; ○, exposed infants that subsequently cleared *Uu* from the trachea [(−) *Uu*]. (*A*) Peak inspiratory pressure (PIP). (*B*) Ventilator rate. (*C*) VEI ($Paco_2$ * rate * PIP/1000). *$p < 0.05$ for (+) *Uu vs* control and (−) *Uu*; #$p < 0.05$ for each group *vs* the others; @$p < 0.05$ for (−) *Uu vs* control and (+) *Uu*. ANOVA with Student-Newman-Keuls *post hoc* analysis.

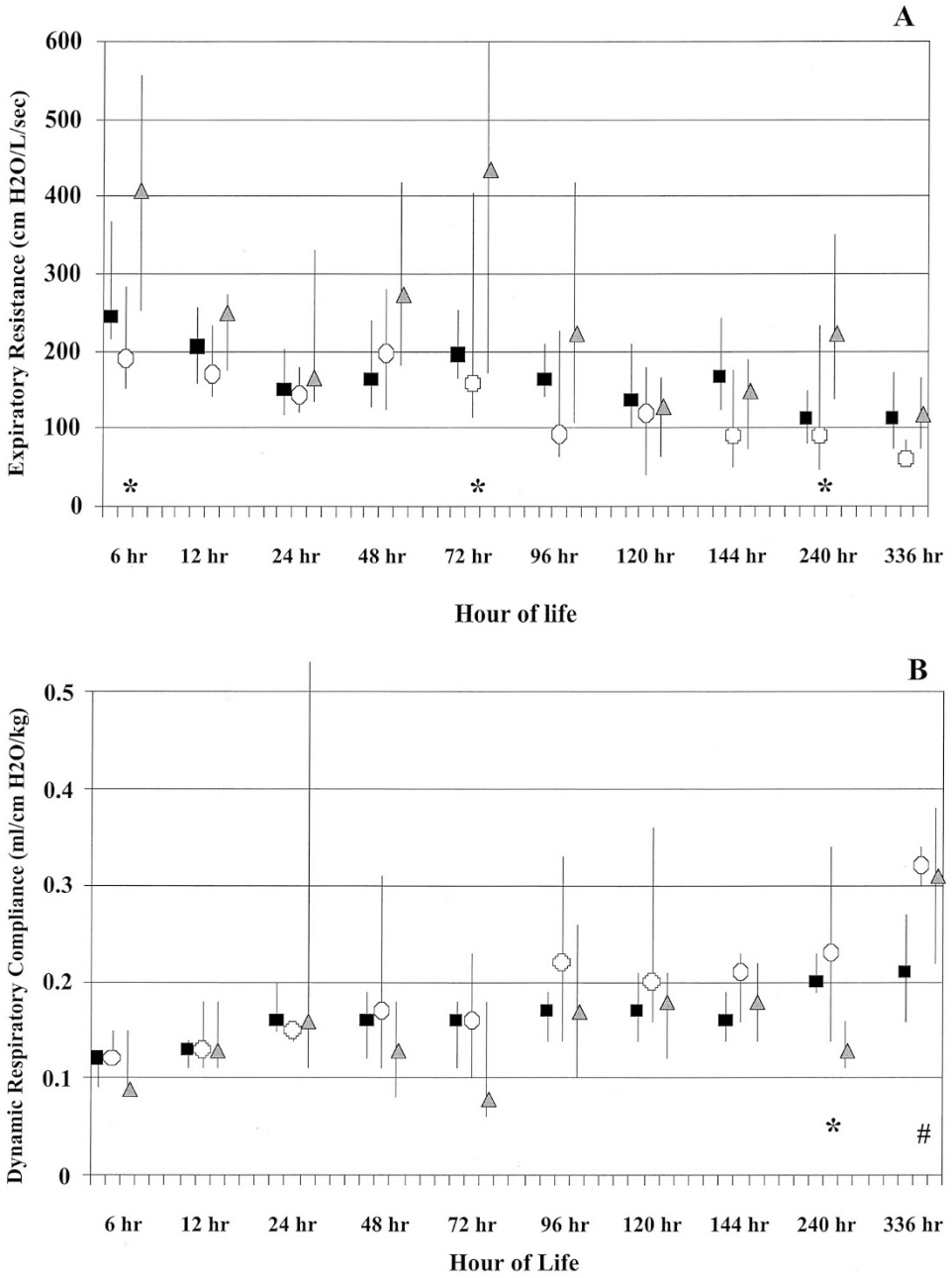

Figure 3. Effect of intra-amniotic *Uu* exposure on pulmonary function tests in 125-d premature baboons. (*A*) Expiratory resistance. (*B*) Dynamic respiratory compliance. Data are expressed as median and quartiles. ■, unexposed controls; △, exposed infants that had persistently positive tracheal cultures [(+) *Uu*]; ○, exposed infants that subsequently cleared *Uu* from the trachea [(−) *Uu*]. *$p < 0.05$ for (+) *Uu vs* control and (−) *Uu*; #$p < 0.05$ for each group *vs* the others. ANOVA with Student-Newman-Keuls *post hoc* analysis.

greater than a mild designation was not found in the control group, whereas several of the *Uu* specimens had moderate to severe microscopic grades (Fig. 4). Control animals showed scattered alveolar macrophages in alveolar/saccular spaces subjacent to bronchioles, whereas the *Uu* groups typically had more numerous alveolar macrophages (Figs. 4 and 5*B*). The most severely affected animal [2F, (+) *Uu*] had more numerous polymorphonuclear neutrophils intermixed with the chronic inflammatory exudate and had the highest TA *Uu* CFU count at necropsy (2.2×10^5; Fig. 5*B*).

DISCUSSION

After 48–72 h of exposure to intra-amniotic *Uu*, we found significant early elevations in tracheal cytokines and white blood cells among all exposed infants compared with unexposed controls. Although early lung function and mechanics were similar between *Uu*-exposed infants and controls, by 48 h of age, two distinct patterns emerged among *Uu*-exposed infants. One group, designated (+) *Uu*, manifested persistent cytokine (IL-6 and IL-8) elevation in TAs accompanied by

Table 3. *IL concentrations from TAs of unexposed controls compared with antenatal Uu-exposed infants by presence of Uu in TAs at necropsy in 125-d premature baboons*

	Control	(−) *Uu*	(+) *Uu*
IL-6 (pcg/mL)	(n = 5)	(n = 5)	(n = 4)
Fetal lung fluid	1181 (930–1365)*	3613 (2430–15,193)	5171 (2535–22,972)
24 h	583 (321–706)*	2306 (1467–2498)	2214 (211–2385)
72 h	666 (348–852)	990 (380–1130)	1356 (1167–2064)†
120 h	791 (189–807)	435 (183–850)	1107 (857–2075)†
240 h	300 (196–463)	491 (134–989)	1043 (775–1350)†
336 h	424 (186–785)	199 (60–1487)	961 (363–1285)
IL-8 (pcg/mL)	(n = 8)	(n = 5)	(n = 4)
Fetal lung fluid	Not available	13,049 (6507–67,568)	18,582 (3910–33,254)
24 h	2527 (169–6255)*	26,179 (11,080–84,206)	33,603 (21,943–57,685)
72 h	8134 (283–17,415)	10,207 (4182–28,832)	50,357 (7200–127,458)†
120 h	6357 (2435–20,098)	8801 (2938–331,682)	30,218 (11,065–62,778)
240 h	3962 (794–14,913)	4264 (249–24,958)	27,050 (6360–79,907)†
336 h	18,176 (1968–50,720)	1336 (1053–10,917)	11,006 (6974–12,713)

Values as median (range).

* $p < 0.05$ by *post hoc* analysis *vs* (−) *Uu* and (+) *Uu.*

† $p < 0.05$ by *post hoc* analysis *vs* control and (−) *Uu.*

Table 4. *Graded lung histopathology for unexposed controls compared with antenatal Uu, exposed infants by presence of Uu in TAs at necropsy in 125-d premature baboons*

	Bronchiolitis*	Pneumonitis	Macrophages*
Controls			
3B	0	0	1
3C	0	1	1
2W	0	1	1
1R	0	1	2
1F	0	0	1
3T	1	1	1
4C	0	1	1
3H	0	1	1
4W	0	1	1
4N	0	1	1
4T	0	1	1
5G	0	0	1
4X	0	0	1
(−) *Uu*			
3G	1	1	1
3X	0	0	1
3Y	1	2	2
1Y	2	0	2
2S	2	1	2
(+) *Uu*			
3F	0	1	1
3Q	1	1	1
4D	2	2	2
2F	2	3	3

* $p < 0.02$ by Mann-Whitney U for controls *vs Uu.* Bronchiolitis and pneumonitis grades: 0, absent; 1, mild, with 1–2 cell layer of mononuclear cells, rare neutrophils; 2, moderate, with > 2 cell layer of mononuclear cells, rare neutrophils; 3, severe, with > 2 cell layer of mononuclear cells, more numerous neutrophils, intermixed intra-alveolar macrophages and scattered neutrophils. Alveolar macrophage grades: 1, minimal, <10 per alveolar space; 2, numerous, >10 in confluent group of alveoli.

significantly worse lung function than control infants. The clinical and radiographic features of these animals were consistent with an acute pneumonitis. Infants with the second pattern, designated (−) *Uu*, showed a reduction in tracheal cytokines and white blood cells after 48 h of age associated with significantly improved lung function compared with control infants. The unique contrast between these two groups of antenatal *Uu*-exposed infants was persistent tracheal colonization in the (+) *Uu* animals out to necropsy at 14 d. After 14 d of mechanical ventilation, (−) *Uu* infants demonstrated improved oxygenation and ventilation compared with controls and (+) *Uu* animals, but there were no differences in pulmonary mechanics or tracheal markers of inflammation between unexposed controls or either group of *Uu*-exposed infants. Histopathologic examination revealed focal airway and saccular inflammation in several of the *Uu*-exposed infants compared with controls. Our study suggests a role for *Uu* in the development of chronic lung injury of some immature infants, but the critical finding is the differential response to *Uu* among an apparently homogeneous group of antenatally exposed, immature, nonhuman primate infants.

Cassell *et al.* (12) first suggested an association between BPD and colonization of the premature infant's lower respiratory tract by *Uu* in 1988. Several subsequent studies also found this association, with a noted predisposition among premature infants <1500 g (13, 20–27). Other reports have not confirmed this correlation, and controversy remains related to the role of lower respiratory tract colonization with *Uu* and subsequent development of chronic lung injury in the premature infant (28–34).

The majority of human studies have relied on cultures obtained within the first 24 h of life for correlation with BPD. Of five studies that followed serial cultures, four found an increased risk for BPD among *Uu*-colonized premature infants, but nasopharyngeal cultures were sometimes used in place of tracheal cultures to determine colonization (13, 22, 23, 34, 35). That 20% to 40% of patients may have positive PCRs for *Uu* with negative cultures also confounds efforts to correlate BPD with colonization by *Uu* (34, 36). Our study demonstrates that approximately 50% of immature baboon infants may clear *Uu* from their pulmonary system within the first week after delivery. Infants who fail to clear *Uu* seem to be at increased risk for early lung dysfunction and injury. These findings mirror a recent report on the natural history of *Uu* colonization in human premature infants (37). In that study, 55% of infants had transient tracheal colonization with *Uu*. More important, in-

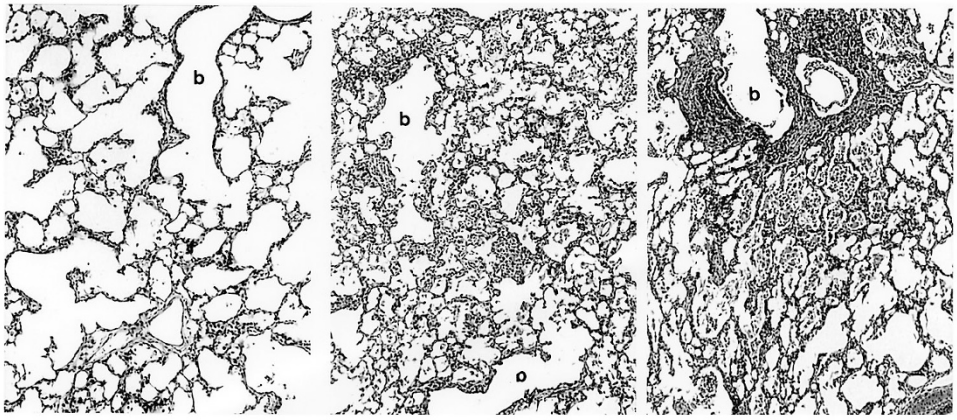

Figure 4. (*A*) The baseline appearance of an uninfected animal ventilated for 14 d: very few inflammatory cells in the bronchiolar (b) wall but more obvious interstitial cellularity in the alveolar walls. (*B*) The lung of a (−) *Uu* animal shows focal bronchiolitis and patchy increased inflammatory cells in the subjacent alveolar walls. (*C*) The lung from the most severely infected animal shows a dense inflammatory cell infiltrate in the bronchiolar was as well as the intra-alveolar and interstitial compartments. Hematoxylin and eosin; original magnification ×40.

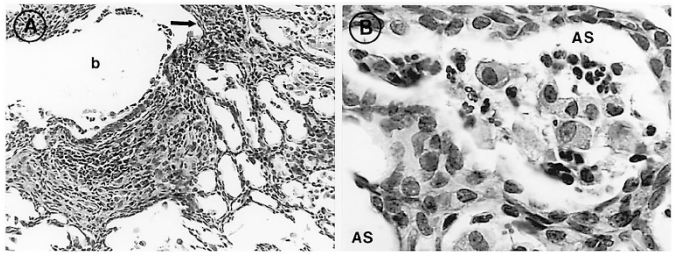

Figure 5. Higher magnification micrographs of the cellular infiltrates in the bronchiolar wall (*A*) and the distal parenchyma (*B*) reveal a dominance of mononuclear inflammatory cells and fewer numbers of polymorphonuclear leukocytes. A few inflammatory cells are seen in the bronchiolar lumen (b). The bronchiolar epithelium is denuded focally (arrow). The wall of the bronchiole contains a striking infiltrate of monocytes, some lymphocytes, and polymorphonuclear leukocytes. The subjacent alveolar walls (*B*) are hypercellular. Focally, an intra-alveolar (AS) exudate of alveolar macrophages, monocytes, and neutrophils is present. Hematoxylin and eosin; original magnification ×100 (*A*) and ×250 (*B*).

fants who had persistent tracheal colonization had a significantly increased risk for BPD.

The duration of antenatal exposure in our study was relatively brief and may not be consistent with actual exposure times in the human situation, in which intrauterine colonization may be present for weeks (38–40). However, all animals had similar initial CFUs of *Uu* in amniotic fluid and fetal lung fluid at the time of birth, as well as from TAs at 24 h of age. In addition, compared with controls, all *Uu*-exposed infants had significantly elevated cytokine and white blood cell values in the amniotic fluid and in TAs at birth and 24 h of age, consistent with an enhanced inflammatory response. Yoon *et al.* (41) reported similar increases in amniotic fluid cytokines and white blood counts in a group of women with preterm premature rupture of the membranes and positive amniotic fluid cultures for *Uu*. We hesitate to conclude on the basis of a single specimen that acute chorioamnionitis, similar to the human condition, consistently occurs in this model.

Several human reports have found *in vivo* evidence for enhanced pulmonary inflammatory response among premature infants colonized with *Uu* at birth (7, 42–44). Groneck *et al.* (35) demonstrated that preterm infants who were infected with *Uu* and subsequently developed chronic lung disease exhibited increased tracheobronchial cytokine levels compared with initially colonized infants who did not develop chronic lung disease. As the enhanced inflammatory response was associated with decreased gestational age, the role of immaturity-related host defense was considered. Given that all of the animals in our study were of similar gestational age, our findings suggest there may be a genetic maturational component to the immune response.

Colonization with a microorganism does not equate to infection. The majority of infants colonized with group B streptococcus do not develop invasive disease. Likewise, many infants may harbor respiratory syncytial virus but not develop significant lower respiratory tract infection. Differences in virulence by serovar subtype, maternal-fetal-neonatal immune system response, and intrinsic pulmonary defense factors, such as surfactant proteins A and D, may contribute to an individual's risk for infection (45–47). The recent findings by Lofgren *et al.* (47) that the risk for severe respiratory syncytial virus infection has a genetic association related to specific surfactant protein A alleles point out the importance of variable host intrinsic immune responses at the genetic level.

Women with amniotic fluid colonization by *Uu* have been found to have significantly higher rates of adverse perinatal outcome, including increased preterm birth rates, higher perinatal mortality, and significant neonatal morbidity (36, 48, 49). It is clear, however, that not all exposed infants have adverse outcomes. In fact, several reports have suggested that chorioamnionitis, especially associated with *Uu*, may result in less respiratory distress syndrome and improved survival rates among immature infants (6, 13, 50). In a series of elegant studies, Jobe and colleagues (51, 52) demonstrated that antenatal endotoxin can enhance surfactant production and acutely improve lung function, although this may be at a cost to later lung development. Our finding that one group of *Uu*-exposed infants manifested a more benign early course of respiratory distress syndrome is consistent with human reports. Given the two divergent clinical pictures that we encountered, it seems

that the maternal-fetal-neonatal immunologic response to antenatal *Uu* may play a critical role. That we did not expose control animals to a sham amniocentesis 48 h before delivery also leaves open the possibility that procedure-related stress may have contributed in a beneficial way to those infants who subsequently cleared the *Ureaplasma* from their airways (53).

The presented model did not result in maternal or neonatal systemic inflammatory changes as measured by serum cytokine and white blood cell counts. It is unclear whether intra-amniotic *Uu* causes a systemic inflammatory response in the human maternal–fetal dyad (54). Although Yoon *et al.* (41) found a significant increase in fetal cord blood IL-6 values in the presence of amniotic fluid *Uu*, they did not report increased maternal serum values. *Uu* is generally considered a weak pathogen except under conditions of immune compromise and may not be capable of generating a systemic inflammatory response in a healthy pregnant woman. As previously noted, the short duration of intrauterine exposure also may not have been long enough to produce a fetal systemic response (38–40).

The pathologic findings in the lung were focal and reflected the findings in only one lobe, the right lower lobe; thus, any disease variability in the entire lung could explain the overlap of the scores. Despite the two significantly different clinical courses, animals from each *Uu*-infected group had histologic features that overlapped those of the control animals. Only one animal had intra-alveolar pathology of intermixed neutrophils and alveolar macrophages, a finding that pathologists would readily diagnose as a focal pneumonitis. The interstitial pneumonitis would be more subtle and bothersome to diagnose on biopsy samples because of the tissue compression frequently observed in such samples. It is the composite of findings, a bronchiolitis with subjacent interstitial pneumonitis and increased alveolar macrophages, that characterizes the *Uu* histopathology in this model. This histologic pattern unfortunately is not unique to *Uu*-induced lung disease but, if identified in a lung biopsy of a neonate, would suggest that appropriate cultures be done to confirm the diagnosis. Unlike the recent reported finding of moderate to severe fibrosis in autopsy lung specimens of human infants with *Uu* pneumonia, significant fibrosis was not present after 14 d of ventilation in our animals (55). As ventilator days in the human study ranged from 7 to 45 d, this difference may be due to augmentation of the fibrotic response related to more prolonged mechanical ventilation or to differences in serovar pathogenicity. The lack of elevated IL-8 values in the bronchoalveolar lavage fluid at the time of necropsy supports the histopathology of *Uu* infection in that intra-alveolar exudates filled with polymorphonuclear leukocytes were not the usual finding in the lung specimens. This pattern is clearly different from what we have observed in other bacterial pneumonias in the baboon in which high levels of IL-8 in bronchoalveolar lavage fluid correlated well with intra-alveolar neutrophil-rich exudates (56).

The finding in (−) *Uu* animals of less clinical lung disease than unexposed controls is consistent with the human situation, in which premature infants with minimal lung disease subsequently develop radiographic and clinical features of BPD (13). The presence of prenatal infection induces a premature recruitment of inflammatory cells and surfactant production, both of which may be abnormal when compared with term animals but that could accelerate lung maturation. In the absence of a sustained tracheal *Uu* presence (by culture or PCR) and decreased tracheal cytokine concentrations, it is difficult to explain which factors contributed to histologic pulmonary inflammation in this group. A synergistic effect between hyperoxia and *Uu* has been demonstrated *in vivo*, and *Uu* induces IL-6 and IL-8 in human neonatal pulmonary fibroblasts even in the absence of hyperoxia (57, 58). A wide variety of cytokines and chemokines may play roles in the inflammatory response. Factors other than the limited cytokines that we measured may be involved (35, 42, 59).

The lack of an acceptable animal model has hampered our ability to better understand any potential role for *Uu* in neonatal chronic lung injury. We have demonstrated that it is possible to produce an intra-amniotic and localized fetal pulmonary inflammatory response in the immature baboon after a relatively brief exposure to *Uu* and that both an acute and a chronic pulmonary response was manifest. Additional studies are needed to determine whether more prolonged exposure can initiate a fetal systemic inflammatory response, to further define factors in the immunologic response that mitigate host colonization, and to broaden the evaluation of other inflammatory mediators/processes. Studies of the effects of *Uu* on other organ systems, most notably the brain, and therapeutic interventions are additional important future goals.

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