Chronic endotoxin exposure does not cause sustained structural abnormalities in the fetal sheep lungs

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Submitted 21 October 2004; accepted in final form 4 January 2005

Kallapur, Suhas G., Ilias Nitos, Timothy J. M. Moss, Boris W. Kramer, John P. Newnham, Machiko Ikekami, and Alan H. Jobe. Chronic endotoxin exposure does not cause sustained structural abnormalities in the fetal sheep lungs. Am J Physiol Lung Cell Mol Physiol 288: L966–L974, 2005. First published January 7, 2005; doi:10.1152/ajplung.00389.2004.—Chronic early gestational chorioamnionitis is associated with development of bronchopulmonary dysplasia in preterm infants. A single intra-amniotic exposure to endotoxin decreased alveolarization and reduced expression of endothelial proteins in 125-day gestational age preterm lambs. We hypothesized that prolonged exposure to intra-amniotic endotoxin would cause progressive lung inflammation and inhibit alveolar and pulmonary vascular development. Endotoxin (1 mg/day) or saline was administered via an intra-amniotic osmotic pump from 80 to 108 days of gestational age (continuous pump) or by four weekly 10-mg intra-amniotic endotoxin injections starting at 100 days of gestational age (multiple dose). Lung morphometry, lung inflammation, vascular effects, and lung maturation were measured at delivery. The continuous pump lambs delivered at 100 days (~70% of total endotoxin exposure) had lung inflammation, fewer sacculles, and decreased endothelial proteins endothelial nitric oxide synthase and VEGF receptor 2 expression compared with controls. The continuous pump (delivered at 138 days) and multiple dose lambs (delivered at 130 and 145 days) had mild persistent lung inflammation and no significant differences in lung morphometry or expression of endothelial proteins compared with controls. Surfactant saturated phosphatidylcholine pool sizes were increased in all endotoxin-exposed groups, but lung function was not changed relative to controls. Contrary to our hypothesis, a prolonged fetal exposure to intra-amniotic endotoxin caused mild persistent inflammation but did not lead to progressive structural abnormalities in lungs of near-term gestation lambs.

chorioamnionitis; bronchopulmonary dysplasia; respiratory distress syndrome; lung maturation; surfactant

FETAL EXPOSURE TO CHORIOAMNIONITIS and increased proinflammatory cytokines in amniotic fluid and cord blood are associated with increased risks of preterm delivery, bronchopulmonary dysplasia and brain injury (37, 43, 44). The chorioamnionitis associated with preterm deliveries before 30 wk of gestation in humans is caused by organisms of low pathogenicity and can be very chronic and clinically unapparent (10). Fetal exposure to chorioamnionitis is associated with a decreased risk of respiratory distress syndrome but with an increased risk of bronchopulmonary dysplasia, which is thought to result from the progression of an inflammatory process that began prenatally (11, 17, 40). Although preterm infants at increased risk of developing bronchopulmonary dysplasia have increased proinflammatory mediators in their airways at birth (39, 40), there is no information about the persistence or progression of lung inflammation during fetal exposure to prolonged chorioamnionitis in the human.

We have developed chorioamnionitis models in fetal sheep to explore how inflammation influences the fetal lung (19, 32). Intra-amniotic injections of Escherichia coli endotoxin or IL-1β in fetal sheep caused acute lung inflammation/injury followed by a lung maturation phenotype within 4–7 days (18, 41). This lung maturation phenotype was characterized by improved lung mechanics and gas exchange, large increases in surfactant lipids and proteins, and increased antioxidant enzymes (18, 38). We recently reported that chronic colonization of sheep amniotic fluid with Ureaplasma urealyticum, a common cause of human chorioamnionitis, also caused lung maturation in preterm lambs delivered at 125 days of gestation (32). However, unlike humans, neither high-dose intra-amniotic endotoxin nor intra-amniotic infection with Ureaplasma urealyticum induces preterm labor in sheep.

The lung maturation induced by intra-amniotic endotoxin was accompanied by decreased expression of vascular endothelial proteins and decreased alveolar development within 7 days of intra-amniotic endotoxin exposure in the preterm lamb lung at 125 days of gestation (20, 42). Similarly, a continuous intra-amniotic exposure to endotoxin from 80 to 108 days of gestation disrupted alveolarization at 125 days gestation (33). Therefore, chorioamnionitis can disrupt microvascular and alveolar development in the preterm fetal lung causing histological changes similar to bronchopulmonary dysplasia (4). It is not known whether the decreased alveolar numbers observed at 125 days gestation persist until later gestation. We hypothesized that a prolonged exposure to intra-amniotic endotoxin would cause a progressive inhibition of alveolar and pulmonary vascular development, as occurs in mice that overexpress proinflammatory cytokines (7, 12, 36, 46). Prolonged fetal exposure to intra-amniotic endotoxin was modeled using two separate protocols: 1) intra-amniotic endotoxin given by osmotic pump for 28 days beginning at 80 days gestation (continuous pump) (33) or 2) four intra-amniotic injections of endotoxin, given at weekly intervals, beginning at 100 days of gestation (multiple dose). Lung morphometry, lung inflammation, vascular effects, surfactant, and lung function were mea-
sured between 130 and 145 days gestation, a developmental
period after alveolarization and microvascular development of
the fetal sheep lung is well established (35).

METHODS

The animal studies were performed in Western Australia using
date-mated Merino ewes with singleton gestations. The studies were
approved by the animal care and use committees of the Department of
Agriculture, Western Australia, and of Cincinnati Children’s Hospital
Medical Center.

Delivery of Intra-amniotic Endotoxin by Osmotic
Pump (Continuous Pump)

We infused 1 mg endotoxin/day (E. coli 055:B5 solubilized in
saline; Sigma, St. Louis, MO) delivered by surgically placing a 28-day
osmotic pump (2ML4; Alzet, Palo Alto, CA) in the amniotic cavity at
80 days gestation (33) (term is 150 days). The osmotic pump was
filled with 2 ml of endotoxin (14 mg/ml) or saline and infused at 3.0
µl/h, resulting in a daily dose of 1 mg. The comparison group received
an equivalent saline infusion from a surgically placed osmotic pump.

Fetal sheep were delivered by cesarean section at 100 days gestation
for evaluation of the inflammatory response after infusion of
endotoxin or saline, and saline-exposed fetuses were delivered at 138
days gestation to assess the residual effects of the endotoxin exposure.

Multiple-dose Intra-amniotic Endotoxin Injections (Multiple Dose)

Date-bred Merino ewes were randomized to 10 mg of endotoxin or
saline injections, each given in a volume of 2 ml by ultrasound-guided
intra-amniotic injection at 100, 107, 114, and 121 days of gestational
age (18). The 10-µg dose was chosen because it gave a consistent
lung maturation response (25), and the injections were begun at 100
days gestation to expose the fetal lung during the saccular and early
alveolar periods of lung development (35). To verify intra-amniotic
rather than allantoic injection, Na+ and Cl− concentrations were
determined on samples of fluid aspirated immediately before endo-
toxin or saline injection (18). Lambs were delivered at 130 and 145
days gestation for evaluations of the fetal lungs. These lambs were not
ventilated after delivery.

Delivery and Ventilation

Each ewe was sedated with ketamine (1 g im) and xylazine (25 mg
im) followed by spinal anesthesia (2% Lidocaine, 3 ml). The fetal
head was exposed through maternal midline abdominal and uterine
incisions, and amniotic fluid was collected. The fetus was sedated (10
mg/kg ketamine im), and, after administering local anesthetic (2%
Lidocaine sc), we performed a tracheotomy and secured an appropri-
ate-sized endotracheal tube. Lung fluid was aspirated by syringe, the
animal was delivered, the umbilical cord was cut, and the animals
were weighed. The umbilical arterial blood samples were collected for
blood gas measurements (Rapid lab 865, Bayer) and for total and
differential white blood cell counts. The animals that were ventilated
received 10 mg/kg ketamine im. The animals that were not ventilated
received a lethal dose of pentobarbital by intravenous injection.

Newborn lambs delivered at 138 days gestation after exposure to
endotoxin or saline by osmotic pumps were ventilated for 40 min to
evaluate lung function as described previously (18). Temperature was
maintained at 39°C with an overhead warmer and plastic wrap. An
arterial catheter was advanced into the descending aorta via an
umbilical artery, and lambs were anesthetized with pentobarbital
sodium (15 mg/kg). Animals were placed on pressure-limited infant
ventilators set to deliver 100% oxygen at a rate of 40 breaths/min, an
inspiratory time of 0.75 s, and a positive end expiratory pressure
(PEEP) of 3 cmH2O pressure. Peak inspiratory pressure (PIP) was
initially set at 35 cmH2O. Tidal volume was monitored continuously
with a neonatal respiration monitor (Acutronic, Baar, Switzerland).
Arterial carbon dioxide partial pressure was measured every 10 min,
and PIP was adjusted to maintain adequate ventilation. Other venti-
lator settings were not altered during the study. Compliance was
calculated by dividing tidal volume by ventilatory pressure (PIP−
PEEP) and then normalized to body weight (in kg). At 40 min postdelivery, animals were deeply anesthetized with pentobarbital
sodium. We degassed the lungs by clamping the endotracheal tube for
5 min. The chest was opened, the lungs were inflated to 40 cmH2O,
and lung volume was determined (18). The lungs were deflated to 5
cmH2O pressure, and the remaining volume was measured. Lung gas
volume was measured similarly following aspiration of fetal lung fluid
for the lambs that were not ventilated.

Processing of Lungs

The lungs were removed from the chest, and each lung was
weighed. We used the left lung for bronchoalveolar lavage (BAL) by
infusing and withdrawing a sufficient volume of saline at 4°C to fully
distend the lungs; this was repeated five times (18). The five BAL
fluid (BALF) washes were pooled. Tissue from the right lower lobe
was frozen in liquid nitrogen for later analysis. The right upper lobe
was inflation fixed by bronchial instillation with 10% formalin at 30
cmH2O pressure.

Cell Counts, Endotoxin Levels in Amniotic Fluid, BALF, and Cord
Blood Cortisol

Endotoxin levels in the amniotic fluid were measured by a limulus
lysate assay (Cambrex, Walkersville, MD). Amniotic fluid was incu-
bated for 30 min at 37°C with 20 µg/ml N-acetyl-L-cysteine, 1 U/ml
neuraminidase, and 20 U/ml hyaluronidase (Sigma) to reduce the
viscosity (34). Cells were isolated from aliquots of amniotic fluid and
BALF by centrifugation at 500 g for 10 min, and the pellets were
resuspended in PBS. After total cell counts using Trypan blue exclu-
sion to identify live cells, differential cell counts were performed on
cytospin preparations stained with Diff-Quick (Dade Behring,
Düdingen, Switzerland). Cortisol was measured in the plasma
obtained from cord blood by a radioimunoassay (MP Biomedici-
cals, Orangeberg, NY).

Lung Morphometry

All morphometric assessments were performed on the right upper
lobe with fixed lung volume measured by volume displacement (42).
Each lobe was then cut into 5-mm serial slices, and three slices were
randomly chosen for morphometric examination. Measurements were
made on each of three 5-µm hematoxylin and eosin-stained sections
per lobe. Digitized images from 10 nonoverlapping parenchymal
fields were captured from each 5-µm section with a Spot RT digital
camera interfaced with a Olympus Bx51 microscope and a computer.
Images were examined at a final magnification of ×850. We counted
the number of points that fell on air space and on alveolar septal tissue
and the number of air/tissue air/intercepts by superimposing a linear
counting grid (40 lines/80 points). In cross section, alveoli
were defined as those structures wholly enclosed by respiratory
epithelium. Relative size and the presence of secondary alveolar septa
distinguished alveolar ducts from alveoli. Size and morphology (wall
thickness and cellularity) were used to distinguish alveoli from sac-
cules (42). Ambiguous structures were rejected. Total number of
alveoli, alveolar wall thickness, and total alveolar surface area in the
right upper lobe were determined by the methods and equations
described previously by our group (42). For the lungs at 100 days
gestation, the saccular structures were similarly measured.

Cytokine and Vascular mRNA

Total RNA isolated from lung tissue and cell pellets was used for
multiprobe RNase protection analysis as described previously (21).
PECAM-1 antibody (sc-1505), rabbit polyclonal anti-Tie-2 antibody (Transduction Labs, Lexington, KY), goat polyclonal antibodies diluted in blocking buffer: mouse monoclonal anti-eNOS with the primary antibody overnight at 4°C using one of the following membranes by electroblotting (Invitrogen). These blots were incubated proteins from the gel were transferred to polyvinylidene difluoride electrophoresis (Novex precast gels; Invitrogen, Carlsbad, CA), and of protein sample per lane were resolved by 3–8% Tris-acetate gel sonicated and centrifuged, and the protein content in the supernatant ing protease inhibitors as described (20). In brief, the samples were evaluations of vascular proteins

Frozen lung samples were homogenized in ice-cold buffer containing protease inhibitors as described (20). In brief, the samples were sonicated and centrifuged, and the protein content in the supernatant was determined by the bicinchoninic acid method. Fifty micrograms of protein sample per lane were resolved by 3–8% Tris-acetate gel electrophoresis (Novex precast gels; Invitrogen, Carlsbad, CA), and proteins from the gel were transferred to polyvinylidene difluoride membrane by electroblopping (Invitrogen). These blots were incubated with the primary antibody overnight at 4°C using one of the following antibodies diluted in blocking buffer: mouse monoclonal anti-eNOS antibody (Transduction Labs, Lexington, KY), goat polyclonal anti-PECAM-1 antibody (sc-1505), rabbit polyclonal anti-Tie-2 antibody (sc-324), or rabbit polyclonal anti-VEGFR2 antibody (sc-504) (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated for 1 h at room temperature with the appropriate conjugated secondary antibody. After washing, bands were visualized by chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Buckinghamshire, UK) and radiographed. The autoradiographs were scanned at high resolution, and images were acquired with Adobe

Table 1. Continuous pump: delivery data

<table>
<thead>
<tr>
<th></th>
<th>Delivery at 100 Days of Gestation</th>
<th></th>
<th>Delivery at 138 Days of Gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
<td>Saline</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>0.93±0.05</td>
<td>0.88±0.03</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>Cord blood pH</td>
<td>7.35±0.02</td>
<td>7.25±0.02†</td>
<td>7.29±0.1</td>
</tr>
<tr>
<td>PO2, mmHg</td>
<td>49±3</td>
<td>61±3†</td>
<td>60±3</td>
</tr>
<tr>
<td>PO2, mmHg</td>
<td>19±2</td>
<td>12±2</td>
<td>14±2</td>
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<tr>
<td>Liver weight, g/kg body wt</td>
<td>42±2</td>
<td>42±2</td>
<td>27±1</td>
</tr>
<tr>
<td>Spleen weight, g/kg body wt</td>
<td>2.1±0.3</td>
<td>2.2±0.2</td>
<td>4.4±1.1</td>
</tr>
<tr>
<td>Neutrophils in cord blood, ×10^9/l</td>
<td>0.52±0.11</td>
<td>1.81±0.31†</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Cord plasma cortisol, µg/dl</td>
<td>0.42±0.04</td>
<td>0.31±0.02</td>
<td>1.1±0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05; †P < 0.01 vs. saline at same gestational age.

The sheep-specific riboprobes for IL-1β, IL-6, IL-8, TNF-α (21), and IL-10 (25) and VEGF, platelet endothelial cell adhesion molecule (PECAM)-1, and Tie-1 (20) have been described. A 990-bp fragment in the sheep Tie-2 coding region was cloned based on homology to the corresponding bovine and mouse sequences using PCR. The resulting plasmid, pGEMT-sTie2, was cut with RsaI, and SP6 RNA polymerase was used to generate an antisense riboprobe (protected fragment 420 bp). The plasmid pKRX-Flk1, a generous gift of Dr. J. L. Segar (University of Iowa), was cut with BsaI, and T7 RNA polymerase was

Table 2. Multiple dose: delivery data

<table>
<thead>
<tr>
<th></th>
<th>Delivery at 130 Days of Gestation</th>
<th></th>
<th>Delivery at 145 Days of Gestation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
<td>Saline</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.1±0.3</td>
<td>3.2±0.1</td>
<td>4.8±0.5</td>
</tr>
<tr>
<td>Lung weight, g/kg body wt</td>
<td>30±2.2</td>
<td>34±1</td>
<td>35±1</td>
</tr>
<tr>
<td>Spleen weight, g/kg</td>
<td>1.8±0.1</td>
<td>2.5±0.3</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Cord plasma cortisol, µg/dl</td>
<td>3.2±2.6</td>
<td>1.6±0.4</td>
<td>8.2±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 1. Continuous pump: lung morphometry. A: surface area for gas exchange. B: saccular/alveolar number. C: saccular/alveolar wall thickness are shown in preterm lambs exposed to continuous intra-amniotic pump infusion of saline (controls) or endotoxin starting at 80 days (d) gestational age and delivered at 100 or 138 days gestational age. Endotoxin infusion decreased saccular numbers at 100 days, but at 138 days no morphometric differences between controls and endotoxin-exposed groups were detected (*P < 0.05 vs. controls).
Photoshop software. Densitometric quantitation was performed with ImageQuant v1.2 software (Molecular Dynamics, Sunnyvale, CA).

Vascular Morphometry

Measurements of arteriolar wall thickness were made using α-smooth muscle actin immunostaining to demarcate the muscularis media (20). Ten arterioles per lamb with measurements of <50 μm external diameter accompanying the terminal bronchioles (identified by morphologic criteria) were measured by a blinded observer, and three to four lambs were evaluated per group. Only transversely sectioned airways were evaluated to minimize distortion of arteriolar muscularis media. Morphometric measurements included thickness of the muscularis media, external vessel diameter, external vessel area, and area of smooth muscle (vessel lumen external area-internal area) and were analyzed using Metamorph version 6.1 software (Universal Imaging, Downingtown, PA) on digitally acquired images. The wall thickness was expressed as [(2× medial wall thickness)/external diameter] × 100.

Saturated Phosphatidylcholine

Lipids were extracted from aliquots of the BALF or lung homogenates with chloroform-methanol. Saturated phosphatidylcholine (Sat PC) was isolated from lipid extracts by neutral alumina column chromatography after exposure to osmium tetroxide (28). Sat PC was quantified by phosphorus assay (2).

Statistical Analyses

The BAL cell numbers and cytokine mRNA are represented as median with range. The other values are given as means ± SE. Normally distributed data were compared between control and treated groups by Student’s t-test or one-way ANOVA, and post hoc pair wise comparisons were made using Dunnett’s procedure. For data not normally distributed, global comparisons were made by nonparametric Mann-Whitney test or Kruskal-Wallis ANOVA on ranks, and post hoc pairwise comparisons were made using Dunn’s procedure. Statistical significance was accepted for \( P < 0.05 \).

RESULTS

Status of Animals at Delivery

Continuous pump. Lambs were delivered at 100 days of gestation to evaluate lung inflammation and morphometry during the endotoxin exposure and at 138 days to assess residual effects on the late-gestation fetal lungs (Table 1). The amniotic fluid endotoxin levels measured at 100 days of gestation were \( 1.9 \pm 0.2 \times 10^4 \) EU/ml in the endotoxin pump group. This endotoxin level is consistent with our previously published amniotic fluid clearance after a single dose of intra-amniotic endotoxin (34). There were no effects on birth weight or lung weight relative to body weight at 100 days gestation after 20 days of endotoxin exposure. However, cord blood pH and \( PO_2 \) were lower, and \( PCO_2 \) was higher for the endotoxin-exposed animals. Other indicators of a systemic effect of the endotoxin exposure were a higher liver weight and increased neutrophils in cord blood. At 138 days gestation, 30 days after the osmotic pump had stopped delivering endotoxin to the amniotic fluid, birth weight was not affected nor were the cord blood gas values. The plasma cortisol values were low in both the 100-day and 138-day saline- and endotoxin-exposed groups.

Multiple dose. The fetal lungs were evaluated 9 and 24 days following the last of the four weekly intra-amniotic doses of endotoxin. At 130 days gestation, birth weights, lung weights, and plasma cortisol values were not different between endotoxin-exposed and control lambs (Table 2). The endotoxin exposure in animals delivered at 145 days did not decrease birth weight, change lung weight, or increase plasma cortisol.
Lung Morphometry

Continuous pump. For the lambs delivered at 100 days, the right upper lobe volumes (ml/kg) were 5.3 ± 0.6 for the saline group vs. 4.8 ± 0.3 for the endotoxin-exposed group (P = 0.5 not significant [NS]). The number of saccules was decreased significantly to 57% of the control value (Fig. 1). Air space surface area or thickness of the saccular walls were not significantly altered. However, in the lambs delivered at 138 days, 30 days after the cessation of endotoxin infusion, no differences in surface area, alveolar numbers, or alveolar wall thickness were detected between endotoxin and control groups. Similarly, the right upper lobe volumes (ml/kg) were 6.0 ± 0.9 for the saline group vs. 5.7 ± 0.5 for the endotoxin exposed group (P = 0.8 NS). Representative sections of the lungs are shown in Fig. 2.

Multiple dose. Compared with controls, in both the groups of endotoxin-exposed animals delivered at 130 and 145 days, no significant changes in surface area, alveolar numbers, or alveolar wall thickness were detected (the alveolar number at 145 days shown in Fig. 3B in the endotoxin group was 30% less than control, P = 0.09). Also the right upper lobe volumes (ml/kg) were 4.9 ± 0.6 for the saline group vs. 4.6 ± 0.6 for the endotoxin-exposed group (P = 0.8 NS). Representative sections of the lungs are shown in Fig. 2.

Lung and Amniotic Fluid Inflammation

Continuous pump. In endotoxin-exposed lambs delivered at 100 days, inflammation was apparent by increased neutrophils in the amniotic fluid and the neutrophils and monocytes in BALF (Table 3). The mRNAs for cytokines TNF-α and IL-10 were modestly increased in lung tissue of endotoxin-exposed 100-day preterm lambs by 3.6 ± 1.0 and 1.5 ± 0.2 times over control values, respectively (P < 0.05). In endotoxin-exposed lambs delivered at 138 days, neutrophils remained increased in the amniotic fluid, and neutrophils and monocytes were increased in the BALF (Table 3). Despite the persistence of inflammatory cells, TNF-α and IL-10 were not different from control values in the lung tissue at 138 days (0.84 ± 0.27 and 0.85 ± 0.22 times control, respectively).

Multiple dose. In endotoxin-exposed lambs delivered at 130 days, inflammatory cells (neutrophils and monocytes) were significantly increased in the BALF with variability between animals (Fig. 4). The mRNA for IL-6, IL-8, and IL-1β were also significantly increased in lung tissue with a large variability between animals. In the 145-day lambs, 24 days after the last endotoxin dose, inflammatory cells remained increased in the BALF although proinflammatory cytokine mRNA values were less elevated in lung tissue than the endotoxin-exposed 130-day group.

Vascular Effects

Continuous pump. Endotoxin-exposed lambs delivered at 100 days had decreased expression of VEGFR2 and eNOS relative to control values (Fig. 5). However, the arteriolar smooth muscle thickness and the lung protein PECAM-1 level were similar between the 100-day endotoxin-exposed and control lambs. Thus the fetal lung had the inflammation and vascular injury response at 100 days gestation. In the lambs delivered at 138 days, the vascular protein VEGFR2, eNOS, and PECAM-1 were not different from control values. Measurements of the percent muscle thickness of the pulmonary arterioles also were 45 ± 1.9% in controls and 47.5 ± 0.4% with endotoxin exposure, values that were not different.

Table 3. Continuous pump: lung and amniotic fluid inflammation

<table>
<thead>
<tr>
<th></th>
<th>Delivery at 100 Days of Gestation</th>
<th>Delivery at 138 Days of Gestation</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>Neutrophils in amniotic fluid, ×10⁹/ml</td>
<td>0</td>
<td>1.3 ± 0.7†</td>
</tr>
<tr>
<td>Neutrophils in BALF, ×10⁹/kg</td>
<td>2.1 ± 1.2</td>
<td>65 ± 44*</td>
</tr>
<tr>
<td>Monocytes in BALF, ×10⁹/kg</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
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</table>

Values are means ± SE. BALF, bronchoalveolar lavage fluid. *P < 0.05; †P < 0.01 vs. saline at same gestational age.

Fig. 3. Multiple dose: lung morphometry. A: surface area for gas exchange. B: alveolar number. C: alveolar thickness is shown in preterm lambs exposed to multiple intra-amniotic doses of saline (controls) or endotoxin starting at 100 days gestational age and delivered at 130 or 145 days gestational age. No morphometric differences between controls and endotoxin-exposed groups were detected.
**Multiple dose.** In the 130-day endotoxin-exposed lambs, multiple mRNA and protein indicators of vascular injury were not different from control values, although there was a small statistically significant decrease in VEGF-188 isoform mRNA (Table 4). In the 145-day endotoxin-exposed lambs, all vascular markers were unchanged except for a small decrease in Tie-1 mRNA.

**Surfactant and Lung Function**

**Continuous pump.** The preterm lambs delivered at 138 days were ventilated to assess lung mechanics and gas exchange. The PIP required for endotoxin-exposed lambs to achieve PCO₂ values comparable to controls was lower, and compliance values were increased (but this effect did not reach statistical significance, \( P = 0.06 \)) (Table 5). Lung gas volumes at 40 cmH₂O pressure and at 5 cmH₂O pressure were not different between groups. The striking difference between the endotoxin-exposed and control lambs was the 3.6-fold increase in BALF surfactant.

**Multiple dose.** In the endotoxin-exposed lambs delivered at 130 days, lung gas volume was increased significantly at 5 cmH₂O pressure, and alveolar surfactant was increased by 10.220.33.1 on October 28, 2017 http://ajplung.physiology.org/ Downloaded from
days of recovery before delivery decreased alveolar septation and this study could be due to differences in the nature of the proinflammatory stimuli (e.g., oxygen, mechanical ventilation). However, another explanation may be that fetuses down-regulate the inflammatory response to a prolonged endotoxin exposure.

There is very little information about the fetal immune responses to a chronic proinflammatory stimulus. The fetal lung contains very low levels of the factors thought to modulate responses to endotoxin such as surfactant proteins A and D, lipopolysaccharide binding protein, mature monocytes, or macrophages relative to adult lung (6, 24, 45). The initial fetal exposure.

### Table 4. Multiple dose: vascular effects

<table>
<thead>
<tr>
<th>mRNA values</th>
<th>Delivery at 130 days of Gestation</th>
<th>Delivery at 145 days of Gestation</th>
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<tr>
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<td>Saline</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>1.0±0.12</td>
<td>1.16±0.11</td>
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<tr>
<td>Tie-1</td>
<td>1.0±0.23</td>
<td>0.89±0.22</td>
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<tr>
<td>Tie-2</td>
<td>1.0±0.14</td>
<td>1.70±0.30</td>
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<tr>
<td>VEGFR2</td>
<td>1.0±0.09</td>
<td>0.96±0.11</td>
</tr>
<tr>
<td>VEGF-165 isofrom</td>
<td>1.0±0.12</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>VEGF-188 isofrom</td>
<td>1.0±0.04</td>
<td>0.78±0.03*</td>
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<th>Protein values</th>
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<th>Endotoxin</th>
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<td>PECAM-1</td>
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<td>1.03±0.13</td>
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<tr>
<td>Tie-2</td>
<td>1.0±0.08</td>
<td>1.14±0.12</td>
<td>1.0±0.06</td>
<td>0.84±0.20</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1.0±0.19</td>
<td>1.21±0.15</td>
<td>1.0±0.06</td>
<td>1.02±0.09</td>
</tr>
<tr>
<td>eNOS</td>
<td>1.0±0.20</td>
<td>1.12±0.34</td>
<td>1.0±0.10</td>
<td>0.72±0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE (relative to saline group, value = 1). PECAM, platelet endothelial cell adhesion molecule; VEGFR2, VEGF receptor 2; eNOS, endothelial nitric oxide synthase. *P < 0.05 vs. saline.

750% (Fig. 6). In the endotoxin-exposed lambs delivered at 145 days, alveolar surfactant also was higher than in the saline-treated control animals. The prolonged endotoxin exposure did not change lung gas volumes at 145 days.

### Table 5. Continuous pump, delivery 138 days: ventilatory and surfactant measurements

<table>
<thead>
<tr>
<th>Values at 40 min of Age</th>
<th>Saline Pump</th>
<th>Endotoxin Pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak inspiratory pressure, cmH₂O</td>
<td>23.6±1.3</td>
<td>20.5±0.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.41±0.03</td>
<td>7.38±0.03</td>
</tr>
<tr>
<td>PCO₂, mmHg</td>
<td>38±2</td>
<td>42±3</td>
</tr>
<tr>
<td>PO₂, mmHg</td>
<td>389±32</td>
<td>455±59</td>
</tr>
<tr>
<td>Tidal volume, ml/kg</td>
<td>9.1±0.5</td>
<td>9.2±0.5</td>
</tr>
<tr>
<td>Compliance, ml/kg/cmH₂O</td>
<td>0.39±0.3</td>
<td>0.45±0.1</td>
</tr>
<tr>
<td>Lung gas volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 cmH₂O, ml/kg</td>
<td>40.6±3.2</td>
<td>41.6±1.3</td>
</tr>
<tr>
<td>5 cmH₂O, ml/kg</td>
<td>26.9±2.2</td>
<td>30.2±2.0</td>
</tr>
<tr>
<td>Sat PC in BALF, μmol/kg</td>
<td>7.4±1.9</td>
<td>27.1±4.7*</td>
</tr>
<tr>
<td>Total lung Sat PC, μmol/kg</td>
<td>62±11</td>
<td>110±24</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sat PC, saturated phosphatidylcholine. *P < 0.01 vs. saline.

![Fig. 6. Multiple dose: lung maturation. A: BALF saturated phosphatidylcholine (Sat PC) pool size expressed per kg body wt. B: lung volumes measured at 5 and 40 cmH₂O pressure in lambs exposed to multiple intra-amniotic doses of saline (controls) or endotoxin starting at 100 days gestational age and delivered at 130 or 145 days gestational age. Exposure to endotoxin increased surfactant Sat PC at 130 and 145 days and lung volume at 130 days (*P < 0.05 vs. controls).](http://ajplung.physiology.org/)
fetal immune responses are modulated by the opposing effects of “tolerance” and “maturation.” However, it is not known whether endotoxin tolerance also occurs in the fetal lung inflammatory cells.

Persistent lung inflammation 9–30 days after intra-amniotic endotoxin in this study was indicated by the presence of inflammatory cells in the airway and increased expression of proinflammatory cytokine mRNA expression in the lung. We have previously reported that lung inflammatory cells can persist up to 25 days after a single intra-amniotic endotoxin exposure, probably due to impaired fetal phagocytosis of apoptotic inflammatory cells (21). In response to a single intra-amniotic endotoxin dose, lung IL-1β is maximally increased ~50-fold at 2 days, and the expression decreases to control levels by 7 days (21). In the present study, there was a wide variability in increases in lung IL-1β by 7 days (21). In the present study, there was a wide variability in increases in lung IL-1β, IL-6, and IL-8 mRNA in the lambs delivered between 130 and 145 days. There was no progressive or additive inflammatory injury to prolonged chronic or repetitive intra-amniotic exposures to endotoxin compared with a single intra-amniotic endotoxin dose. The inflammation was modulated without an increase in the counterregulatory cytokine IL-10. Similarly, another counterregulatory mechanism, an increase in endogenous cortisol, did not occur. Recent studies from adult mice demonstrate that counterregulation of the signaling molecules in the Toll-like receptor pathway mediates endotoxin tolerance (22), but whether these factors operate in the preterm is not known.

Previous results from our laboratory demonstrated a 28–42% decrease in alveolar numbers and inhibited microvascular development 7–17 days after intra-amniotic endotoxin exposure and delivery at 125 days (33, 42). In this study, preterm lambs delivered at 100 days after 20 days of intra-amniotic endotoxin exposure had inflammation, hypoxia, and fetal acidosis. These factors might have contributed to the anatomic and vascular changes consistent with disrupted lung development (43% reduction in saccular numbers in 100-day gestation endotoxin-exposed lambs). We previously reported decreased alveolar numbers at 125 days of gestation following a continuous endotoxin exposure (0.6 mg/day) from 80 to 108 days of gestation (31). In this study, a higher daily endotoxin exposure (1 mg/day) from 80 to 108 days of gestation did not result in persistent structural abnormalities at 138 days, suggesting that inadequate endotoxin exposure does not explain the different results. Therefore, the surprising new finding is that the fetal lung recovers and develops relatively normally despite prolonged endotoxin exposure when assessed closer to term (alveolar numbers tended to be lower in the endotoxin-exposed group compared with controls delivered between 130 and 145 days). We previously reported that a single dose of endotoxin had no residual effects 2 mo after term birth (30). The mechanism by which the fetal lung repairs the lung injury response and displays “catch up” despite chronic exposure to proinflammatory mediators is unknown. Some of the variables in this “recovery” response may be gestational age of assessment, gestational age during exposure, and magnitude of the inflammatory responses. Small differences may not be apparent given the relatively small numbers of animals in the study groups. In these experiments, the exposures were primarily during the canalicular and saccular stages with the osmotic pump or the saccular and early alveolar stages with the weekly exposures. The lung inflammatory responses, though persistent, were mild and not progressive. The assessments later during the alveolar stage showed no significant residual lung remodeling.

Decreased angiogenic growth factors, endothelial proteins, and pulmonary hypertension are a central feature of bronchopulmonary dysplasia (1, 3, 13, 14, 26). Previous results from our laboratory demonstrated decreased angiogenic and endothelial protein expression and arteriolar smooth muscle hypertrophy in 125-day preterm lambs 4–7 days after endotoxin exposure (20). In this study, although VEGFR2 and eNOS expression decreased in endotoxin-exposed 100-day gestational lambs compared with controls, there were no changes in the arteriolar smooth muscle thickness. Consistent with the alveolar/saccular surface area, the vascular surface area in both the 100- and 138-day gestation saline- and endotoxin-exposed groups were also similar as indicated by the PECAM-1 protein levels. Similar to the alveolar morphometry, neither decreased endothelial protein expression nor arteriolar smooth muscle changes could be demonstrated in 130- to 145-day gestation endotoxin-exposed lambs. The mechanisms by which the fetal lung modulates alveolar and vascular recovery despite chronic exposure to proinflammatory mediators remain to be determined.

There are clinical implications of these experimental results. About 70% of the preterm infants delivered after preterm labor before 30 wk of gestation have histological chorioamnionitis (10). Much of this chorioamnionitis is caused by organisms of low pathogenicity such as ureaplasma and mycoplasma species that may be present for weeks or months before the preterm delivery. In sheep, ureaplasma can persist in amniotic fluid for months and can induce lung inflammation and maturation (32). The results from the study suggest that the fetus exposed to a chronic proinflammatory milieu may not have persistent progressive structural lung abnormalities. However, the fetal lung exposed to a prolonged antenatal proinflammatory stimulus may respond to postnatal proinflammatory stimuli differently than a naïve lung. We previously showed that mechanical ventilation-induced lung injury is amplified when exposed to endotoxin 30 days but not 4 days before preterm delivery (15, 16). Also, infants exposed to a prolonged antenatal proinflammatory stimulus may be at risk for brain injury (27). Thus the interactions between antenatal and postnatal inflammation on the lung and other organ systems must be complex and need further study.

GRANTS

This work was funded by National Institutes of Health Grants K08 HL-70711 (to S. G. Kallapur), HD-12714 and HL-65397 (to A. H. Jobe) and by the Women and Infants Research Foundation of Western Australia.

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