Initial responses to ventilation of premature lambs exposed to intra-amniotic endotoxin 4 days before delivery

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Ikegami, Machiko, Suhas G. Kallapur, and Alan H. Jobe. Initial responses to ventilation of premature lambs exposed to intra-amniotic endotoxin 4 days before delivery. Am J Physiol Lung Cell Mol Physiol 286: L573–L579, 2004. First published November 14, 2003; 10.1152/ajplung.00211.2003.—Preterm delivery is frequently preceded by chorioamnionitis, resulting in exposure of the fetal lung to inflammation. We hypothesized that ventilation of the antenatally inflamed lung would result in amplification of the lung injury. Therefore, we induced fetal lung inflammation with intra-amniotic endotoxin (10 mg of Escherichia coli 055:B5) 4 days before premature delivery at 130 days of gestation. Lung function and lung inflammation after surfactant treatment and 4 h of mechanical ventilation were evaluated. 

Methods

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continuously during ventilation (model CP-100, Bicore Monitoring Systems, Anaheim, CA). Vt was measured with a Fleisch pneumotachometer at 30 min, 1 h, and every hour thereafter. PIP and FIO₂ were regulated to maintain a target P CO₂ of 55 mmHg and a target P O₂ of 150–200 mmHg to minimize possible effects of the patent ductus arteriosus. A 5-Fr catheter was advanced into the aorta via an umbilical artery, and filtered fetal blood (10 ml/kg) collected from the placenta was transfused within 10 min of delivery. Dynamic compliance was calculated as Vt (ml) normalized to body weight (kg) and divided by the ventilatory pressures (PIP – PEEP; cmH₂O) (16). The ventilatory efficiency index (VEI) was calculated as follows: VEI = 3,800/(respiratory rate) × (PIP – PEEP) × P CO₂, where 3,800 is a CO₂ production constant (28). The arterial catheter was used for blood gas analysis and blood pressure monitoring. Fluid containing 10% dextrose was infused through a leg vein at 4 ml·kg⁻¹·h⁻¹. Rectal temperature was monitored and maintained at 38–39°C, the normal body temperature for sheep, with heating pads and radiant heat. Supplemental ketamine and acepromazine were given to prevent spontaneous breathing. After 4 h, each animal was deeply anesthetized with pentobarbital sodium (25 mg/kg iv), and FlO₂ was changed to 1.0. The endotracheal tube was clamped for 3 min to permit O₂ absorption, and the animal was exsanguinated.

**Pressure-volume curves and lung processing.** The thorax of each lamb was opened, the lungs were inflated with air to 40 cmH₂O for 1 min, and a maximum lung volume was recorded (24). The pressure was sequentially lowered to 20, 15, 10, 5, and 0 cmH₂O, and lung volumes (ml/kg body wt) were recorded after 30 s at each pressure. Volumes were corrected for the compliance of the system. Left and right lung weights were recorded. Pieces of the right lower lobe were immediately frozen in liquid nitrogen for RNA isolation. BAL of the left lung was done with 0.9% NaCl at 4°C, and the process was repeated five times (21). The BAL fluid (BALF) was pooled, and aliquots were saved for measurement of total protein (23), cytokines, cell number and differential count, and H₂O₂ production. With the use of left-to-right lung weight ratios, results using BALF were calculated for the total lung and normalized to kilogram body weight.

Amniotic fluid was incubated for 30 min at 37°C with 20 mg/ml N-acetylcycteine and 55 U/ml hyaluronidase (Sigma) to reduce the viscosity (21). Amniotic-acetylcysteine and 55 U/ml hyaluronidase (Sigma) to reduce the viscosity (21). Amniotic-acetylcysteine and 55 U/ml hyaluronidase (Sigma) to reduce the viscosity (21). Amniotic-acetylcysteine and 55 U/ml hyaluronidase (Sigma) to reduce the viscosity (21). Amniotic-acetylcysteine and 55 U/ml hyaluronidase (Sigma) to reduce the viscosity (21).

**Cytokine mRNA.** Total RNA was isolated from tissue from the right lower lobe and from cell pellets of BALF by guanidinium thiocyanate-phenol-chloroform extraction. RNA protection assays were performed with total RNA from lung tissue and cell pellets (18). Briefly, cRNA transcripts of ovine interleukins (IL-1β, IL-6, and IL-8) and ovine ribosomal protein L32 as a reference RNA were synthesized with [32P]UTP (Life Sciences Products, Boston, MA) using SP6 or T7 polymerase (RNase protection assay 111, Ambion, Austin, TX). Aliquots (10 μg) of RNA were incubated with excess radiolabeled probes for cytokines and L32 at 35°C for 18 h. The remaining single-stranded RNA was digested with RNase A/T1 (Ambion). Protected fragments were electrophoresed on a 6% polyacrylamide-urea sequencing gel and visualized by autoradiography. Densities of the protected bands were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant Software (Molecular Dynamics). ELISAs were used to measure IL-1β, IL-6, and IL-8 in the supernatant of centrifuged lung homogenate and concentrated BALF (14). IgG fractions prepared from rabbit anti-sheep IL-1β, IL-6, or IL-8 serum were the primary antibodies, and guinea pig anti-sheep IL-1β, IL-6, or IL-8 serum was the secondary antibody. Antibodies for IL-6 and IL-8 were purchased from Chemicon International (Temecula, CA), and antibody for IL-1β was made in our laboratory. Standard curves were constructed from the absorbance of known amounts of recombinant sheep cytokines (custom-made by Protein Express, Cincinnati, OH). Standard curves were sensitive at 0.1–80 ng/ml for IL-1β, 0.15–50 ng/ml for IL-6, and 1.5–100 ng/ml for IL-8, with a correlation coefficient of 0.99 for all assays.

**Immunohistochemistry for cytokines.** The right upper lobe was inflation fixed at 30 cmH₂O pressure with 10% formalin. Paraffin-embedded 5-μm-thick tissue sections were treated for antigen retrieval using 0.1 M citrate, and endogenous peroxidase activity was inactivated by incubation with H₂O₂. Nonspecific binding sites were blocked with serum. Lung sections were incubated with a rabbit polyclonal antibody for ovine IL-6 (Chemicon), mouse polyclonal antibody for ovine IL-8 (Chemicon), or rabbit polyclonal antibody for ovine IL-1β (14). After the slides were washed with PBS to remove unbound antibody, they were incubated with the secondary biotinylated antibody against rabbit (for IL-6 and IL-1β) or mouse (for IL-8) IgG (Vector Laboratories, Burlingame, CA). After addition of avidin (Vector Laboratories), staining of positive cells was developed with diaminobenzidine and cobalt with a nuclear fast red counterstain.

**Data analysis.** Values are means ± SE. Analysis of variance followed by the Student-Newman-Keuls multiple comparison procedure was used for comparisons of the four groups. Two-tailed unpaired t-tests were used for two-group comparisons. Significance was accepted at P < 0.05.

**RESULTS**

**Physiological status.** The lambs in the four groups had similar birth weights (Table 1). The amniotic fluid from the lambs exposed to endotoxin had increased numbers of inflammatory cells. The number of white blood cells (WBC) in cord blood samples was similar in the four groups, whereas after 4 h of ventilation the number of WBC in the endotoxin-exposed group was significantly higher than in the control group. The lambs also had similar blood pressures and heart rates throughout the ventilation period, with the 4-h values given in Table 1. Although the pressures used to ventilate the endotoxin-exposed lambs were 4.4 cmH₂O higher than those used to ventilate

<table>
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<tr>
<th>Table 1. Description of lambs</th>
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<tr>
<td>No Vent</td>
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<tr>
<td>Control (n = 6)</td>
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<tr>
<td>Birth wt, kg</td>
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<tr>
<td>Inflammatory cells in amniotic fluid, ×10⁶/ml</td>
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<tr>
<td>WBC, ×10³/ml</td>
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<td>Vt, ml/kg</td>
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<td>P50/FIO₂</td>
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<td>SBP, mmHg</td>
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Values are means ± SE. No Vent, unventilated; 4-h Vent, ventilated for 4 h; WBC, white blood cells; Vt, tidal volume; P50/FIO₂, ratio of P50 to inspired O₂ fraction; SBP, systolic blood pressure; HR, heart rate. *P < 0.05 vs. control.
control lambs ($P < 0.05$; Fig. 1B), $P_{CO_2}$ values (Fig. 1A), gas exchange as evaluated by the VEI (Fig. 1C), and compliance values (Fig. 1D) were similar for the 4-h ventilation period. Oxygenation as measured by $P_{O_2}/F_I_{O_2}$ and $V_T$ were similar at 4 h (Table 1). More volume was retained in the pressure-volume curves of the two groups of ventilated lambs than the unventilated lambs (Fig. 2). There were no significant differences between the two unventilated groups. The increased volumes with ventilation were anticipated, because the ventilated lambs were treated with surfactant and the lungs had been opened by the mechanical ventilation. For the two ventilated groups, volumes at 5 and 10 cmH$_2$O on the deflation limbs of the pressure-volume curves were higher for the endotoxin-exposed group than for the control group. Small differences were seen in ventilation pressures ($P_{IP}$ – PEEP), pressure-volume curves, and WBC between the ventilated control and endotoxin groups. These small differences were not of physiological importance, and the general conclusion was that the antenatal endotoxin exposure had little effect on postnatal lungs or the general physiological status of the animals.

**Inflammation in the lungs.** In the unventilated control lambs, no neutrophils and very few monocytes were recovered by BAL (Fig. 3). Both cell types were increased in the endotoxin-exposed lambs that were not ventilated. Ventilation for 4 h recruited $1.0 \pm 0.3 \times 10^6$ neutrophils/kg to the BALF of the control lambs. Ventilation did not further increase the neutrophils in the BALF of the endotoxin-exposed animals from the unventilated values of $56 \pm 19 \times 10^6$ neutrophils/kg. There are no differences in monocyte numbers in BALF between the groups with and without ventilation. H$_2$O$_2$ production by the cells from the BALF was low for unventilated lambs. Ventilation increased H$_2$O$_2$ production in the control and endotoxin-exposed lungs, and qualitatively more H$_2$O$_2$ was produced by the cells from the ventilated and O$_2$-exposed group. Total protein in BALF of the control unventilated lambs was $6.3 \pm 1.4$ mg/kg, and the increase with endotoxin exposure to $12.7 \pm 3.1$ mg/kg was not significant. The protein values for the ventilated lambs were $17.0 \pm 1.7$ mg/kg without endotoxin and $21.8 \pm 3$ mg/kg with endotoxin, and both values were higher than those for unventilated controls ($P < 0.05$). The antenatal endotoxin exposure caused lung inflammation, but the added stimulus of ventilation did not result in increased cell numbers or alveolar protein.

**Immunohistochemistry for cytokines.** Expression of IL-1$\beta$, IL-8 (Fig. 4), and IL-6 was analyzed by immunohistochemistry for three sections for each lamb. The immunostaining pattern and intensity for IL-1$\beta$ and IL-8 were similar for the lambs within each group, and the representative photomicrographs for IL-8 are shown in Fig. 4. IL-1$\beta$ and IL-8 staining was not seen in control lambs (Fig. 4, A and B). IL-1$\beta$ and IL-8 were found in inflammatory cells in the air space for endotoxin-exposed lambs only (Fig. 4, C and D). Ventilation for 4 h (Fig.
4D) did not change the immunostaining for IL-1β or IL-8 relative to the unventilated endotoxin-exposed group (Fig. 4C). IL-6 was not detected in any of the lung tissues (data not shown).

Cytokine mRNAs and proteins. The mRNA for IL-1β was increased fourfold in cells from BALF by the antenatal endotoxin but did not increase further after 4 h of ventilation (Fig. 5). IL-8 mRNA in the BALF cells was not increased significantly by ventilation or endotoxin, and IL-6 mRNA was not detected in the BALF cells. In contrast, ventilation and antenatal endotoxin increased the three cytokine mRNAs, but there was not a further increase with the combination of endotoxin and mechanical ventilation.

The cytokine proteins IL-1β, IL-8, and IL-6 were measured in the supernatant of centrifuged lung tissue homogenates and expressed as amount per kilogram body weight (Fig. 6, A–C). The amounts of IL-1β and IL-8 were significantly increased by antenatal endotoxin exposure. Cytokine proteins were also
measured in concentrated BALF. IL-6 was not detectable in the BALF of any of the groups. IL-1β was detectable in four of the samples from endotoxin-exposed lambs. IL-8 in BALF was significantly higher in unventilated endotoxin-exposed and ventilated endotoxin-exposed groups than in controls (Fig. 5B). Mechanical ventilation did not increase the amounts of the cytokines in the lung homogenate and BALF of the control lambs. The combination of mechanical ventilation and antenatal endotoxin resulted in increased IL-8 in lung homogenate relative to the unventilated control or endotoxin-exposed groups.

**DISCUSSION**

We anticipated that the chorioamnionitis-mediated lung inflammation/injury would result in augmented lung inflammation with the second stimulus of mechanical ventilation. Although qualitatively lung function was slightly worse, higher ventilation pressure was required, and indicators of inflammation tended to be increased, with the only significant increase being IL-8 protein in the lung homogenate, which was not large. Therefore, the superposition of ventilation on the acute phase of lung inflammation/injury caused by endotoxin-in-
duced chorioamnionitis resulted in minimal increased injury within 4 h. In retrospect, this result was similar to the general clinical experience that most infants delivered after chorioamnionitis do not have severe lung disease unless they are septic or have severe pneumonia. However, the interpretation of the clinical information is clouded by the uniform lack of information as to the time of initiation and progression of the chorioamnionitis (9). Prolonged chorioamnionitis may induce lung maturation and further confound an assessment of its importance on lung function after preterm delivery (30, 32). We delivered these lambs 4 days after the injection of endotoxin, because the lung maturation response has not occurred by day 4 (16).

Intra-amniotic endotoxin causes chorioamnionitis and lung inflammation that can be detected within 5 h and persists for ≥15 days (17, 18, 21). The highest number of granulocytes are in BALF at 3 days, and the highest expression of the proinflammation cytokines IL-1 and IL-8 is on day 2 (18, 20). Apoptosis is highest on day 1, and proliferation is evident by day 3 (20); similar responses occur after 4–20 mg of intra-amniotic endotoxin (21). In this experiment, 10 mg of endotoxin were given 4 days before preterm delivery for evaluation of the postnatal response to mechanical ventilation of lungs that are just beyond the peak of injury as indicated by apoptosis and proinflammatory cytokine expression. The preterm lambs were treated with surfactant before ventilation to avoid severe ventilator-mediated injury and to reflect clinical practice (12). Protein levels in BALF were low as a result of surfactant treatment and gentle ventilation (24, 31). The ventilation period of 4 h was selected, because acute inflammation as indicated by granulocyte recruitment and cytokine expression occurs within 4 h and was decreased by 7 h in ventilated preterm lambs (27).

In a previous report, we found that chorioamnionitis induced in sheep 30 days before preterm delivery resulted in minimal residual inflammation in the lungs (13). However, in contrast, mechanical ventilation induced a large increase in monocytes and lymphocytes, but not in granulocytes, in the BALF. This pattern of response was consistent with a memory response that favored innate host defenses over an augmented acute inflammatory response. Ventilation of adult rats exposed to endotoxin resulted in large increases in proinflammatory cytokines, but those effects were elicited by using ventilation styles known to severely injure the lung (5). Dreyfuss et al. (8) recently reviewed the cytokine responses to mechanical ventilation in adult lungs and commented that acute inflammatory responses are not consistently observed. For this experiment, we treated the lambs with surfactant at delivery and limited ventilation pressures and VT to minimize lung injury and to better reflect current clinical practice (12). The lack of an amplified inflammatory response to the combination of endotoxin-induced chorioamnionitis and mechanical ventilation may also have resulted from the normal response of the fetal lung to suppress inflammation within 4 days of the fetal endotoxin exposure (17). The major difference between these experiments is the history of inflammatory exposure, where exposure 30 days before ventilation caused an “immune” response with increased monocytes and lymphocytes, whereas ventilation superimposed on a lung that was resolving inflammation had little effect.

In humans and animal models with inflamed lungs, high-molecular-weight substances such as endotoxin and cytokines can translocate from the lungs to the systemic circulation and cause a systemic inflammatory response (22). This translocation occurred in adult animals only when excessive ventilation was used (26). In contrast, endotoxin appeared in the circulation of surfactant-treated preterm lambs with gentle mechanical ventilation comparable to that used in this experiment, but hyperventilation of the full-term lung was required for intra-thecal endotoxin to have systemic effects (19). Thus the preterm lung was uniquely sensitive to developing shock and falling WBC counts when endotoxin or recombinant IL-1α was instilled into the airways. These endotoxin-exposed animals had modestly increased cytokine levels and high numbers of neutrophils in the BALF at birth. However, systemic WBC did not decrease and blood pressure was similar to that of the control lambs. The half-life of endotoxin in amniotic fluid was ~1.7 days, and the lung inflammation resulted from direct contact of the lung with the endotoxin (25). The physiological stability of the animals indicated that <10 μg of the endotoxin could have entered the systemic circulation from the lungs with mechanical ventilation (19).

The effects of the antenatal endotoxin exposure on lung function, lung inflammation, or systemic responses were evaluated for only 4 h, because by design we were exploring responses to initial lung adaptation after birth. Very little inflammation was induced in this model of gentle ventilation in lambs not exposed to endotoxin. The tendency for increased inflammation may be an indicator that the antenatal inflammation would result in persistent inflammation with continued mechanical ventilation. Such an effect is predicted by the association of chorioamnionitis and mechanical ventilation with BPD (30).

GRANTS
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