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Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs

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Kallapur, Suhas G., Karen E. Willet, Alan H. Jobe, Machiko Ikegami, and Cindy J. Bachurski. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol* 280: L527–L536, 2001.—The inflammatory and lung maturational effects of intra-amniotic exposure to endotoxin were assessed in fetal lambs. Five hours to 25 days after intra-amniotic injection of endotoxin, preterm lambs were delivered at 119–125 days gestation. Intra-amniotic endotoxin caused an inflammatory cell infiltration in amnion/chorion at 5 h, which persisted for 25 days. At 5–15 h after endotoxin, amnion/chorion cytokine mRNAs increased [12- to 26-fold for interleukin (IL)-1β, IL-6, and IL-8 mRNA and 3-fold for tumor necrosis factor-α mRNA]. At 1–2 days after endotoxin, lung cytokine mRNAs increased 6- to 49-fold. Endotoxin caused modest changes in peripheral white blood cell counts and no significant cytokine mRNA responses in fetal liver, placenta, or jejunum. Lung maturation, as characterized by increased lung volumes and alveolar saturated phosphatidylcholine, occurred at 7 days and persisted for 25 days after endotoxin. We conclude that exposure to a single dose of intra-amniotic endotoxin causes inflammation and increases in cytokine mRNA in amnion/chorion and the fetal lung before lung maturation, consistent with the hypothesis that proinflammatory cytokines signal lung maturation.

surfactant; cytokine; respiratory distress syndrome; bronchopulmonary dysplasia

MICROBIAL INVASION OF THE amniotic fluid is present in ~10–20% of women in preterm labor with intact membranes and in ~30–60% of patients with preterm premature rupture of membranes (19, 42, 48). Chorioamnionitis is associated with elevations of the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 in the amniotic fluid (2), and amniotic fluid samples that contain elevated IL-6 predict preterm delivery (20, 49). Thus the epidemiological associations of chorioamnionitis and preterm birth are compelling. Chorioamnionitis is also associated with an increased incidence of chronic lung disease and adverse neurodevelopmental sequelae such as intraventricular hemorrhage and periventricular leukomalacia (1, 19).

The association of chorioamnionitis with respiratory distress syndrome (RDS), however, is not straightforward. Although preterm delivery is the major risk factor for the development of RDS, Watterberg et al. (46) found that chorioamnionitis decreased the risk for RDS but increased the risk for bronchopulmonary dysplasia (BPD) in preterm infants. *Ureaplasma urealyticum* is the most common cause of chorioamnionitis (52). Preterm infants <28 wk gestation with *U. urealyticum* colonization at birth had a lower incidence of RDS but higher incidence of BPD compared with infants who were not colonized (21).

Experimental evidence supports the clinical observations of decreased RDS following exposure to chorioamnionitis. Bry et al. (6) demonstrated that intra-amniotic injection of recombinant IL-1α increased steady-state mRNA levels for surfactant proteins and improved compliance of preterm rabbit lungs. Similarly, in preterm lambs intra-amniotic injection of recombinant IL-1α improved postnatal lung function without increasing fetal cortisol, catecholamines, or peripheral white blood cell numbers (10). The proinflammatory stimulus of intra-amniotic endotoxin given to preterm lambs improved lung compliance and increased surfactant lipids and surfactant protein mRNAs and proteins (27). These maturational effects evaluated 7 days after intra-amniotic endotoxin occurred with only modest inflammation in fetal membranes and lungs (27). The time relationships between cytokine expression, indicators of inflammation and the occurrence of pulmonary maturation after intra-amniotic endotoxin are not known. We hypothesized that intra-amniotic endotoxin would cause chorioamnionitis, which would lead to a cascade of signaling events, resulting in biochemical and physiological markers of lung maturation. We measured the inflammatory cell responses in the amniotic/chorionic membranes and lung and proinflammatory cytokine expression in amnion/chorion, lung, liver, jejunum, and

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placenta at intervals from 5 h to 25 days after intra-amniotic endotoxin injection.

MATERIALS AND METHODS

Animals. The animal studies were performed in Western Australia with date-mated Merino ewes with singleton gestations. The studies were approved by the appropriate animal care and use committees in Australia and at Children's Hospital Medical Center (Cincinnati, OH) One dose of 20 mg of *Escherichia coli* 055:B5 endotoxin (Sigma) or the same volume of a saline control was given by intra-amniotic injection using ultrasound guidance and with electrolyte analysis of amniotic fluid to verify intra-amniotic injection (27).

Delivery and sample collection. Five hours to 25 days after intra-amniotic injection, the ewes were preanesthetized with ketamine and xylazine and received spinal anesthesia for cesarean section delivery of preterm lambs at 119–125 days gestation (term is 150 days). Lambs were delivered through a uterine incision and were given 100 mg/kg pentobarbital sodium by injection via an umbilical vein. The cord was cut and the animal was weighed. Blood was drawn from the umbilical artery for a blood gas, pH analysis and white blood cell (WBC) and differential counts. A roll of amnion and chorion was sampled for snap freezing and for fixation in 4% paraformaldehyde for histopathology. A placental cotyledon was stripped of membranes and slices were snap frozen for RNA analysis.

The chest of the lamb was opened, and a 4.5-mm endotracheal tube was tied into the trachea for measurement of lung gas volume by inflation of the lung with air to 40 cmH_2O pressure (25). The lung was removed and weighed, and the left lung was used for alveolar lavage (27). A piece of the left lower lobe was used for analysis of saturated phosphatidylcholine (Sat PC). Pieces of parenchyma from the center of the right lower lobe were snap frozen for subsequent analyses of mRNA. The right upper lobe was inflation fixed for histopathology. Tissue samples from the liver and jejunum were also snap frozen.

Processing of alveolar wash. Sequential alveolar lavage fluid samples from the left lung were pooled and the volume was measured. A total cell count on the alveolar wash was determined by hemocytometer using trypan blue exclusion to identify live cells. Ten milliliters of alveolar wash were centrifuged at 170 *g* for 5 min, and the cell pellet was resuspended in 1 ml of PBS, of which 100 μl was used for cytospin. The cytospin slides were stained with Leishman's stain (BDH, Poole, UK) for a differential cell count of 200–300 cells. For Sat PC measurements, aliquots from the pooled lavage samples and homogenized pieces of the left lower lobe were extracted with chloroform and methanol (2:1) (23). The lipid extracts were treated with osmium tetroxide, and saturated phosphatidylcholine was recovered after alumina column chromatography and quantified by phosphorus assay (5, 36). The total protein in alveolar washes was measured using the Lowry et al. (35) assay.

Histology of lung and amnion/chorion. Right upper lobes were inflation fixed with 4% paraformaldehyde at 30 cmH_2O pressure. Amnion/chorion membrane rolls were also fixed in 4% paraformaldehyde. Umbilical cord was not included in the tissue sampled. Each piece of lung was cut into 5-mm transverse slices, and three slices per lobe were randomly chosen and paraffin embedded for light-microscopic assessment. Influx of inflammatory cells into the tissues was graded by scoring three 5-μm hematoxylin- and eosin-stained sections as zero (no inflammatory cells), 1 (few), 2 (moderate influx of cells) or 3 (extensive influx of inflammatory cells). Multiple fields in sections from amnion/chorion were similarly scored. A mean score was calculated for each animal. All assessments were made by the same observer (K. E. Willett) in a blinded fashion.

Cloning of sheep cytokines and antisense RNA probes. The sheep cytokines IL-1β, IL-6, and TNF-α were cloned from an adult sheep cDNA library (Stratagene, La Jolla, CA) and IL-8 from adult sheep spleen cDNA by PCR using primers synthesized from published cDNA sequences. Identity of all the clones were confirmed by double-strand sequence comparison with published sequence. The following vectors were used: pCR 2.1 vector (Invitrogen, Carlsbad, CA), pGEM-T and pGEM-7Zf(+) (Promega, Madison, WI). A portion of sheep ribosomal protein L32 cDNA (27) was subcloned into pGEM-7Zf(+) and was used as a reference to standardize for gel loading. Table 1 shows the details of the cloning strategy and generation of RNA probes for the RNase protection assay.

RNase protection assay. Total RNA was isolated as described (3), and 10 μg of total RNA were used for the multiprobe RNase protection assay. Solution hybridization was performed in 80% deionized formamide, 0.4 M NaCl, 2 mM EDTA, and 40 mM PIPES, pH 6.6, using a molar excess of

Table 1. *Cloning of sheep cytokine cDNAs and generation of antisense RNA probes for RNase protection assays*

Insert cDNA	Cloning Strategy	Antisense RNA Probe	Protected Fragment Size, bp	Reference No.
IL-1β	802-bp fragment, nucleotides (33–834) cloned into pCR2.1; this was cut with *Eco*R1 and subcloned in *Eco*R1 site of pGEM-7Z+(pGEM-7Z-IL-1β)	pGEM-7Z-IL-1β linearized with *Sty*I, T7 RNA polymerase	284	12
TNF-α	702-bp fragment, nucleotides (4–706) cloned in pCR2.1; a 535-bp fragment generated by *Eco*RI and *Bsa*I was subcloned in pGEM-7Z+ cut with *Eco*RI and *Sma*I (pTNF 535/1).	pTNF 535/1 linearized with *Ava*II, SP6 RNA polymerase	121	53
IL-6	386-bp fragment, nucleotides (193–578) cloned in pGEM-T (pGEM-IL-6)	pGEM-IL-6 cut with *Bst*N1, SP6 RNA polymerase	185	9
IL-8	152-bp fragment, nucleotides (43–194) cloned in pGEM-T (psIL-8.2/3)	pSIL-8.2/3 linearized with *Not*I, T7 RNA polymerase	152	43
L32	*Apa*LI, *Bam*HI fragment of 397-bp sheep L32 clone (pGEM-sL32) was subcloned in *Sma*I site of pGEM-7Z+(psL32-306/6)	psL32-306/6 linearized with *Bgl*II, T7 RNA polymerase	85	27

IL, interleukin; TNF-α, tumor necrosis factor-α.

[α-P^{32}]UTP-labeled riboprobes for 16 h at 55°C. RNase digestion and recovery of protected fragments was performed using the RPA III kit (Ambion, Austin, TX). Probes for IL-1β, IL-6, IL-8, and L32 were combined and analyzed together. The TNF-α probe was used in a separate hybridization with L32 control probe. To decrease the background when using TNF-α probe, RNase inactivation and extraction were performed using the Riboquant assay system (PharMingen, San Diego, CA). The protected fragments were resolved on 6% polyacrylamide 8 M urea gels, visualized by autoradiography, and quantified on a phosphorimager using ImageQuant version 1.2 software (Molecular Dynamics, Sunnyvale, CA).

Statistics. Inflammatory scores in amnion/chorion and lung were expressed as median with interquartile, 5th and 95th percentile range. The other results are expressed as means ± SE. Comparisons between endotoxin-exposed animals and controls were made with two-tailed unpaired *t*-test, two-tailed Mann-Whitney nonparametric test, or two-way Kruskal-Wallis nonparametric ANOVA as appropriate. Significance was accepted at $P < 0.05$.

RESULTS

Description of lambs. In control animals, saline was injected intra-amniotically 1, 4, or 7 days before delivery. In experimental animals (4–5 animals per group), intra-amniotic endotoxin was given from 5 h to 25 days before delivery. Control and endotoxin-injected lambs were delivered between 119 and 125 days gestation. There were no differences in body weights, cord blood gases, or pH between experimental and control groups.

Inflammatory response in amnion/chorion. In control animals, a few resident macrophages were seen. Leukocyte infiltration in the amnion/chorion was detected within 5 h after exposure to endotoxin (Fig. 1*A*). Inflammatory cell infiltration persisted until 25 days after endotoxin exposure (Fig. 1*B*). Thus intra-amniotic endotoxin caused a rapid and sustained leukocytic infiltration of the fetal membranes.

Cytokine response in amnion/chorion. Cytokine mRNA expression was measured in total RNA isolated from membrane rolls harvested at delivery. This included RNA from cells of amnion, chorion, and infiltrating inflammatory cells in the membranes. In control preterm lambs, there was a low abundance of proinflammatory cytokine mRNAs (Fig. 2*A*). Within 5 h after intra-amniotic endotoxin exposure, the cytokine mRNAs were maximally induced in amnion/chorion. IL-1β, IL-6, and IL-8 mRNA were elevated over control values an average of 14-, 12-, and 24-fold, respectively, at 5 h postendotoxin (Fig. 2*B*). In contrast, TNF-α mRNA expression was elevated only threefold over the control value at 5 h postendotoxin. The cytokine mRNA levels remained elevated at 15 h and decreased to control values by 2 days, even though inflammatory cell infiltrates remained elevated through 15 days. Thus intra-amniotic endotoxin caused a rapid and robust elevation of IL-1β, IL-6, and IL-8 and, to a lesser extent, an increase in TNF-α mRNA expression.

Cytokine response of fetal lung. Expression of cytokine mRNA in the lungs of control lambs was minimal (Fig. 3*A*). At 5 h postendotoxin, all the cytokine mRNAs were increased compared with controls (Fig. 3*B*). In contrast to amnion/chorion where the peak cytokine mRNA expression was detected at 5 h, maximal cyto-

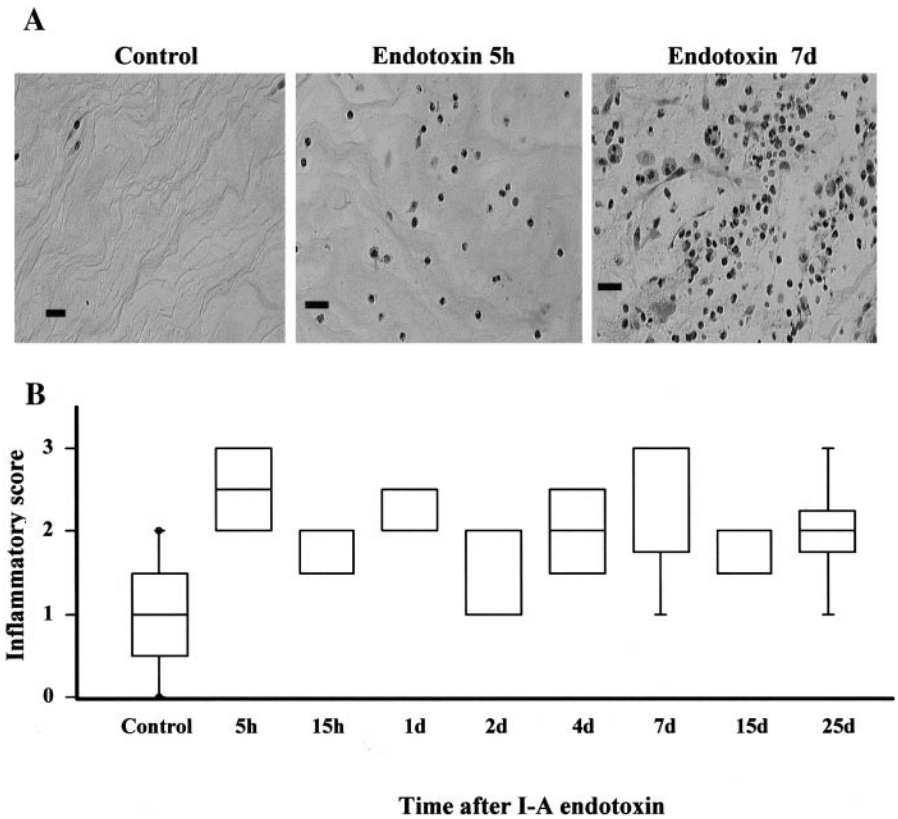

Fig. 1. Intra-amniotic (I-A) endotoxin causes chorioamnionitis. *A*: representative hematoxylin- and eosin-stained sections of amnion/chorion from control and endotoxin-treated animals. Control animals had a few inflammatory cells (score = 1), whereas endotoxin-treated animals at 5 h had moderate inflammatory cell infiltration (score = 2), and endotoxin-treated animals at 7 days had regions with extensive influx of inflammatory cells (score = 3). Most of the inflammatory cells in endotoxin-treated animals were neutrophils by morphology (bar = 20 μm). *B*: plot showing median, 25th and 75th percentile range in a box and 5th to 95th percentile range as error bars. Outliers in the data set are plotted as open circles. At some time points, due to decreased variance in the sample, the median and/or the 5th and 95th percentile coincided with the 25th or 75th percentile. Endotoxin caused an influx of inflammatory cells within 5 h, and this inflammation persisted for 25 days after endotoxin administration. $P < 0.05$ for endotoxin-treated vs. control animals by 2-way Kruskal-Wallis nonparametric ANOVA; $n = 12$ animals for control and 4–5 animals for each time point; d, day(s).

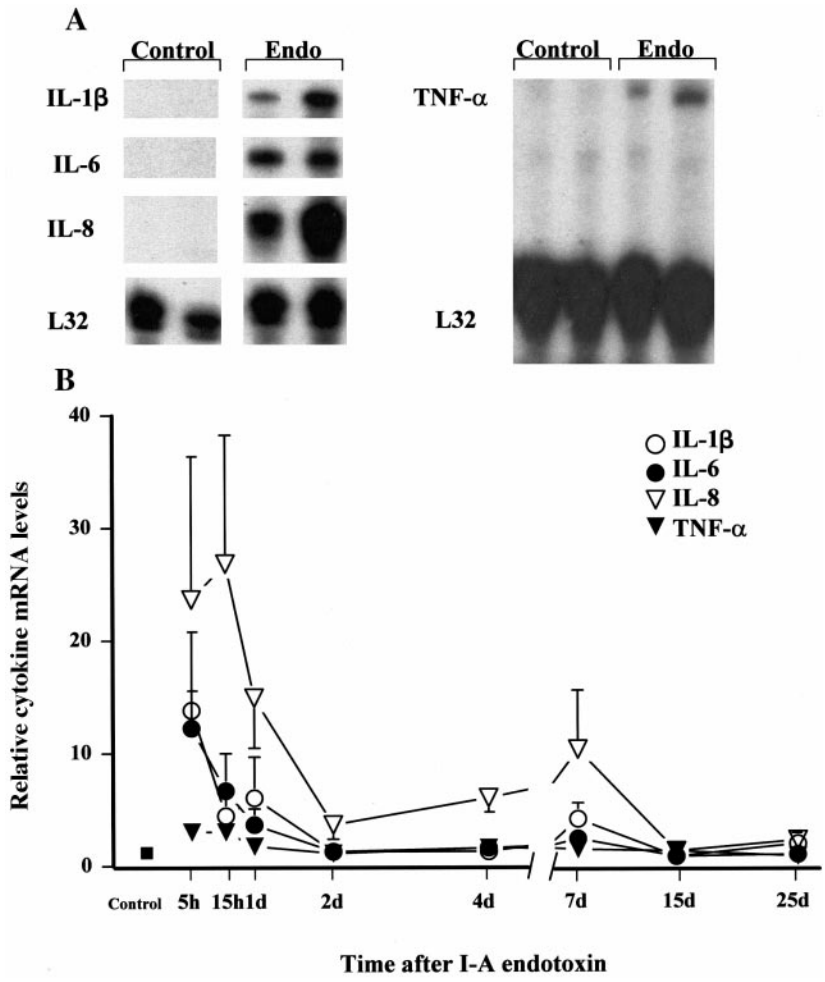

Fig. 2. Time course of endotoxin-induced cytokine mRNA production in amnion/chorion. *A*: representative RNase protection assay of 10 μg of total RNA from amnion/chorion of 2 animals exposed to endotoxin (Endo) or saline (Control) for 5 h are shown. Control animals had low-level cytokine mRNA expression, and within 5 h, peak induction of cytokine mRNA was observed. *B*: cytokine mRNA values were normalized to L32 (ribosomal protein RNA) and plotted as means ± SE. The mean cytokine mRNA signal in control animals was designated as 1 (■), and levels at each time point are expressed relative to controls. Endotoxin increased cytokine mRNA within 5 h, which returned to control values by 2 days; $n = 11–12$ animals for control and 4–5 animals for each time point; $P \leq 0.005$ for all cytokine mRNAs at 5 h vs. controls by Mann-Whitney test. IL, interleukin; TNF-α, tumor necrosis factor-α.

kine expression occurred in the lungs between 1 and 2 days postendotoxin. At 2 days postendotoxin, IL-1β mRNA was elevated 49-fold, IL-6 increased 7-fold, IL-8 increased 9-fold, and TNF-α increased 6-fold. The increased cytokine mRNAs returned to control values by 7–15 days. Thus the preterm lung responded to intra-amniotic endotoxin with elevations of IL-1β, IL-6, IL-8, and TNF-α mRNA expression, which peaked after the expression in the amnion/chorion.

Lung inflammation. Inflammation in the lungs was evaluated by blinded scoring for inflammatory cells in sections of the right upper lobe, cell count with differential counts in the alveolar wash, and measurement of protein in the alveolar washes. No granulocytes were seen in alveolar washes of control animals (Fig. 4*A*). The granulocyte population in the alveolar wash increased to 46×10^4/ml 2 days post-intra-amniotic endotoxin. At 4 days, the granulocytes in alveolar wash increased further and were too numerous to count in the dilution used for cytospin preparations. Alveolar wash granulocytes decreased by 7 days, and at 15 and 25 days, atypical cells were present. These atypical cells did not exclude trypan blue and most likely were dying inflammatory cells. Sections of lung were examined for granulocyte infiltration and quantified by an inflammatory cell score (Fig. 4*B*). The inflammatory score was increased 15 h after endotoxin relative to controls, peaked at 2 days, and slowly declined thereafter. Although the inflammatory scores were elevated, no frank areas of inflammatory infiltrate or consolidation were seen. Consistent with the time course of appearance of inflammatory cells, alveolar wash protein was increased twofold over controls at 2 days but not at other time points after endotoxin ($P < 0.05$ vs. controls). Thus 20 mg of intra-amniotic endotoxin caused a modest lung inflammatory response.

Surfactant and lung gas volumes. Lung gas volumes measured at 40 cmH_2O were significantly increased 2.3-fold at 7 days, 4.5-fold at 15 days, and 3-fold at 25 days after endotoxin relative to control values (Fig. 5*A*). Alveolar Sat PC pool sizes increased 26-fold at 7 days, 77-fold at 15 days, and 28-fold at 25 days after endotoxin relative to those in controls (Fig. 5*B*). The total lung Sat PC pool increased about 1.5-fold at 4 days, 3.2-fold at 15 days, and 1.8-fold at 25 days post-endotoxin (Fig. 5*C*). The increased alveolar Sat PC pool size correlated with larger lung gas volumes. The increased Sat PC pool size and lung gas volumes 7–25 days after treatment was not due to changes in lung weights, since the lung-to-body weight ratio was identical in endotoxin and control animals (0.037 ± 0.001 vs. 0.037 ± 0.001). Thus one intra-amniotic injection of

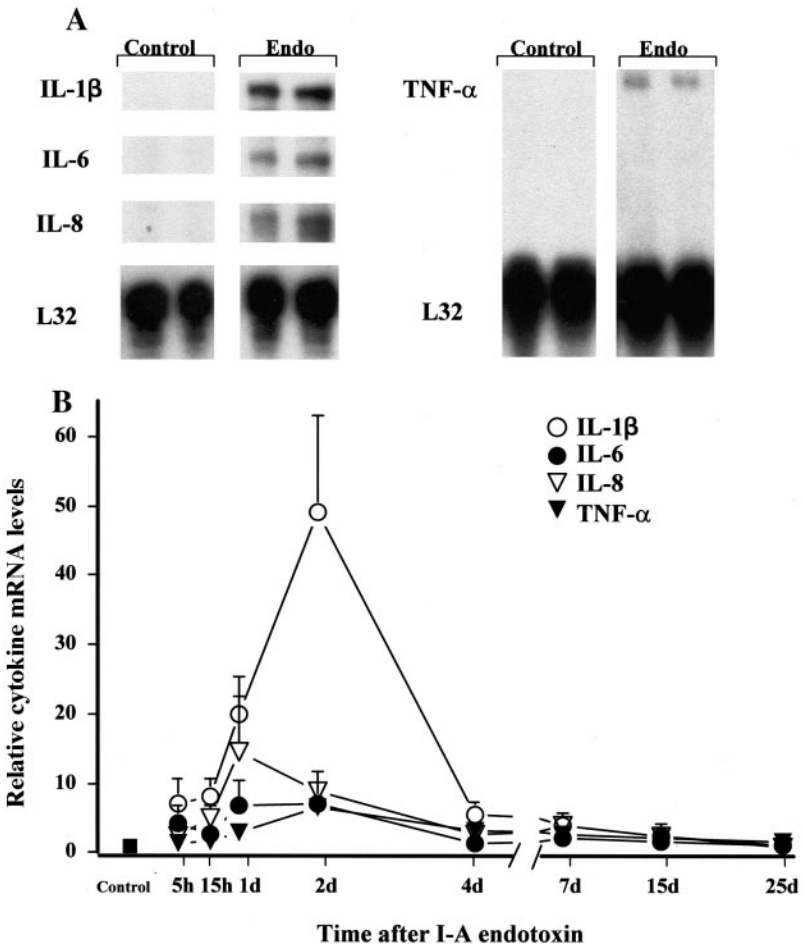

Fig. 3. Delayed induction of cytokine mRNAs in lung. *A*: representative RNase protection assay of 10 μg of total RNA from lungs of 2 animals exposed to endotoxin (Endo) or saline (control) for 1 day are shown. Control animals had low-level cytokine mRNA expression, and induction of cytokine mRNA was apparent at 1 day. *B*: cytokine mRNA values were plotted as means ± SE as in Fig 2. Intra-amniotic endotoxin induced cytokine mRNAs in lung by 5–15 h. Peak levels were measured at 1–2 days; n = 9–12 animals for control and 4–5 animals for each time point; $P \leq 0.005$ for all cytokine mRNAs at 1 and 2 days vs. controls by Mann-Whitney test.

endotoxin caused biochemical and physiological maturation of lungs by 7 days, with the largest effects at 15 days after endotoxin.

Systemic inflammation. One and two days after intra-amniotic endotoxin, the total fetal circulating WBC count decreased from control values. Subsequently, the WBC count increased on *days 4* and *7*, with a return to control levels by *day 15* (Fig. 6). Neutrophils increased about fivefold over control to 2.5×10^6 cells/ml at 7 days after endotoxin. There was a trend for lymphocytes to decrease at 1–2 days after endotoxin. Monocytes increased 4 days after endotoxin. Thus intra-amniotic endotoxin caused a systemic response as indicated by changes in peripheral WBCs.

Cytokine response in liver, placenta, and jejunum. To determine whether there was a generalized induction of cytokines in response to intra-amniotic endotoxin, we measured proinflammatory cytokine mRNA in the fetal liver, placenta, and jejunum. In control and endotoxin-treated animals, there was no detectable expression of TNF-α in these tissues. The livers of control animals had detectable expression of IL-1β mRNA. Five hours after endotoxin, there was a twofold elevation of IL-1β (Fig. 7*A*). Compared with the responses in amnion/chorion or lungs, the liver response was much more modest. In control lambs, expression of IL-6, IL-8, and IL-1β mRNA could be detected in placenta. However, there was no elevation of these cytokine mRNAs in the placenta after intra-amniotic endotoxin exposure (Fig. 7*B*). The control lambs had low levels of mRNA expression for IL-1β, IL-6, and IL-8 in the jejunum, and the cytokine mRNA levels were not elevated in endotoxin-treated lambs relative to controls (Fig. 7*C*). In contrast to the response seen in amnion/chorion and lungs, intra-amniotic endotoxin did not cause cytokine mRNA elevations in liver, placenta, or jejunum.

DISCUSSION

These experiments were performed to characterize a model for the association between chorioamnionitis and induced lung maturation. A 20-mg intra-amniotic endotoxin dose caused chorioamnionitis, lung inflammation, and cytokine mRNA induction in amnion/chorion and lung in fetal sheep. Lung maturation was seen starting at 4–7 days and persisted for 25 days after endotoxin. The cytokine mRNA induction in amnion/chorion and lung was shorter in duration compared with inflammatory changes in these tissues. Distinct temporal patterns in the tissue cytokine mRNA expression profile and lung maturation were seen after intra-amniotic endotoxin exposure. Amnion/chorion cytokine mRNA peak response was detected by 5–15 h after

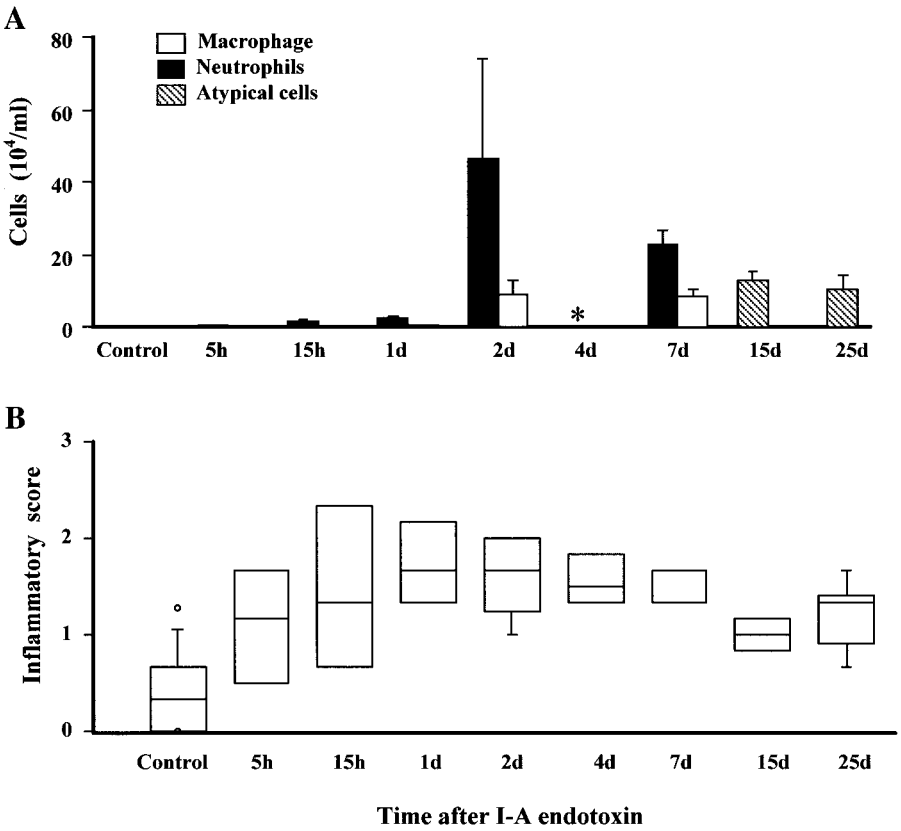

Fig. 4. Intra-amniotic endotoxin caused lung inflammation. *A*: granulocytes, macrophages, and atypical cells were quantified in cytospin preparations from alveolar wash. Inflammatory cells were increased at 2 days, consisting of predominantly neutrophils. At 4 days, there were too many cells to count in the cytospin dilution, as indicated by an asterisk. At 15 and 25 days, atypical cells that were probably dying or dead inflammatory cells were seen. *B*: box plot of inflammatory scores were depicted as in Fig. 1. The median, 25th, and 75th percentile range of data points are shown in a box and 5th to 95th percentile range as error bars. Outliers in the data set are plotted as open circles. At some time points, due to decreased variance in the sample, the median and/or the 5th and 95th percentile coincided with the 25th or 75th percentile. Endotoxin caused an influx of inflammatory cells within 15 h, which was highest at 2 days and decreased by 7 days, but mild inflammation persisted for 25 days after endotoxin administration. $P < 0.05$ for endotoxin-treated vs. control animals by 2-way Kruskal-Wallis nonparametric ANOVA; $n = 12$ animals for control and 4–5 animals for each time point.

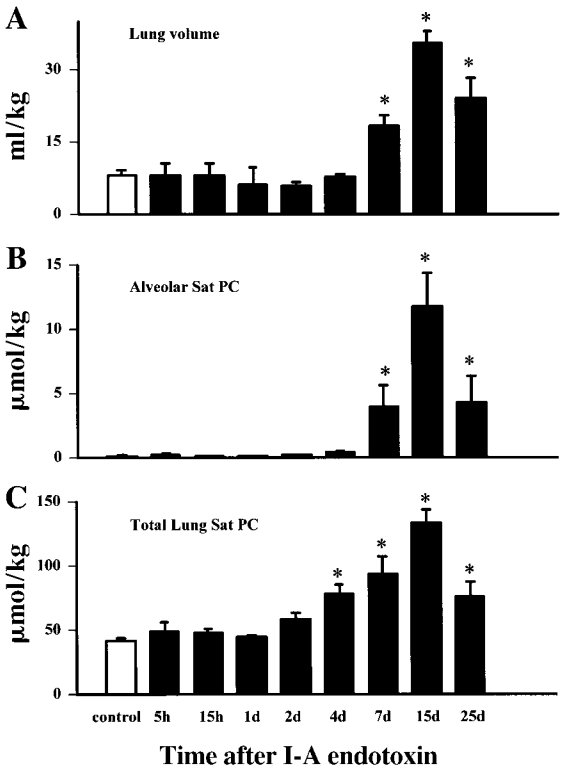

Fig. 5. Intra-amniotic endotoxin induced lung maturation. *A*: maximal lung volumes measured at 40 cmH_2O pressure increased 7, 15, and 25 days after endotoxin. Saturated phosphatidylcholine (Sat PC) in alveolar wash (*B*) and total lung (*C*; alveolar wash plus lung tissue) increased after endotoxin. All values are expressed per kg body wt. $^*P < 0.05$ vs. control by 2-tailed unpaired *t*-test; $n = 12$ animals for control and 4–5 animals for each time point.

endotoxin, whereas the peak lung cytokine mRNA response occurred at 1–2 days after endotoxin. The IL-8 mRNA increase was the most prominent cytokine mRNA response detected in amnion/chorion, whereas IL-1β mRNA induction was the predominant lung response. Lung tissue Sat PC increases were seen 4 days after endotoxin. There was no large systemic inflammatory response to intra-amniotic endotoxin. The results of these experiments are consistent with the hypothesis that the proinflammatory cytokine response associated with chorioamnionitis may be the initial maturational signal to the lung.

In this in vivo experimental model, intra-amniotic endotoxin caused chorioamnionitis, elevated proinflammatory cytokine expression in amnion/chorion, and fetal lung maturation similar to findings in preterm infants (2, 46). The similarities are remarkable because the preterm lambs were exposed to a single dose of endotoxin, whereas preterm infants with chorioamnionitis are frequently exposed to bacterial endotoxins in a prolonged and indolent manner (40). This animal model differs from infants with chorioamnionitis in the lack of systemic effects in lambs and the exposure of the human fetus to live organisms. There was a modest systemic WBC response to intra-amniotic endotoxin and there was no mortality in the lambs, while in preterm infants up to 50% of those exposed to chorioamnionitis can have systemic inflammation (19).

The mechanism of chorioamnionitis-induced lung maturation is unknown. Intra-amniotic injection of IL-1α induces surfactant protein mRNAs and improves lung compliance in preterm rabbits and sheep (6, 10).

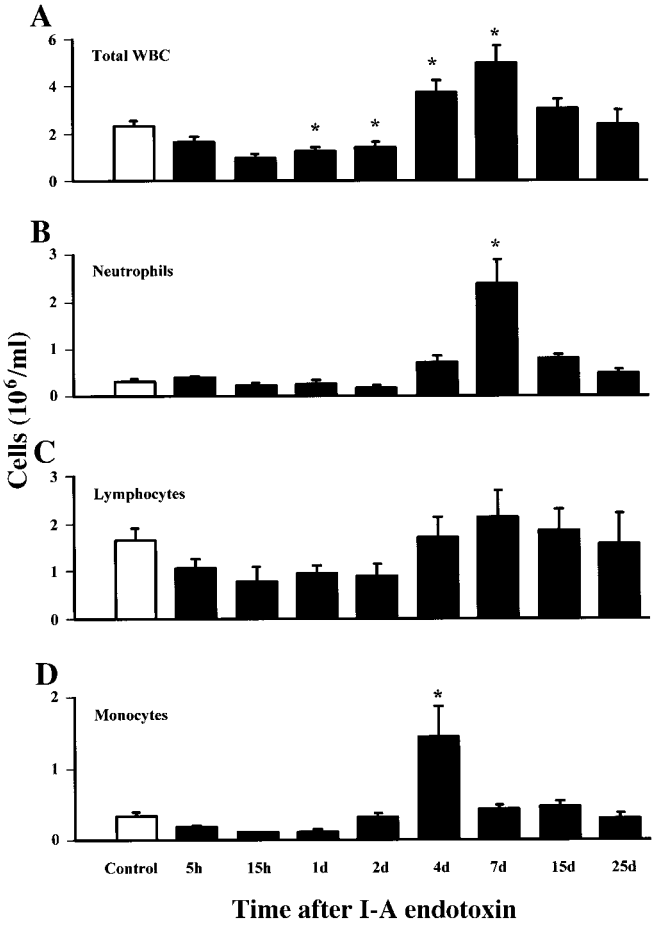

Fig. 6. Effects of intra-amniotic endotoxin on peripheral white blood cells (WBC). Total and differential white blood cell counts from peripheral blood were changed by intra-amniotic endotoxin. *$P <$ 0.05 vs. control by 2-tailed unpaired t-test; n = 12 animals for control and 4–5 animals for each time point.

High-dose IL-1α also increases indicators of lung maturation in 22-day explants of fetal rabbit lung (18). Clinical studies demonstrate the association of histological and bacteriological chorioamnionitis with decreased incidence of respiratory distress syndrome in preterm neonates (21, 46). *Ureaplasma* is a common cause of chorioamnionitis, and even though the organism lacks a cell wall, chorioamnionitis with ureaplasma is associated with increased amniotic fluid TNF-α, IL-1β, and IL-6 levels (52). While a mouse model for increased IL-1 expression in the lung has not been reported, transgenic mice overexpressing IL-6 or TNF-α in the lung have abnormal alveolar architecture and inflammation (7, 39). Thus the experimental evidence suggests that cytokines can modulate lung maturation.

The direct cytokine response of amnion/chorion to endotoxin has been evaluated in vitro in explant and cell culture. Endotoxin causes human amnion/chorion from term gestation fetuses of nonlaboring mothers to increase IL-1β, IL-6, IL-8, and TNF-α secretion into the medium 3- to 10-fold over baseline within 8–12 h after exposure (15). Gram-negative bacteria stimulate more cytokine release by cultured amnion cells than do gram-positive bacteria (41). IL-1β and TNF-α induce IL-6 and IL-8 expression in human amnion/chorion cells, and mRNA increases are seen at 2–4 h after treatment (8, 33, 44). Endotoxin stimulates human amnion and chorion cells to express mRNAs for IL-1β, IL-6, IL-8, and TNF-α in vitro, with differences in the patterns of expression of the cytokines in amnion or chorion (13, 14, 38). In the present in vivo study, cytokine protein levels were not measured due to lack of appropriate reagents. The highest expression of IL-6, IL-1β, and TNF-α mRNAs was detected at 5 h after endotoxin and IL-8 peak mRNA expression was sustained 5–15 h after endotoxin treatment. The in vitro studies suggest that the initial endotoxin effect on amnion/chorion cytokines can be further amplified by the interactions between these cytokines. The sites of cytokine mRNA synthesis in vivo remain to be determined.

In the present study, lung cytokine mRNA was beginning to increase within 5 h, with peak levels reached at 1–2 days after intra-amniotic endotoxin. By comparison, direct intratracheal instillation of endotoxin in adult mice causes peak elevations of proinflammatory cytokine mRNA by 2 h (28). The lung could respond initially to a systemic signal or to granulocytes that are activated and migrate to the lungs. WBCs accumulate in the lungs of preterm infants soon after delivery and ventilation (11). It is possible that the

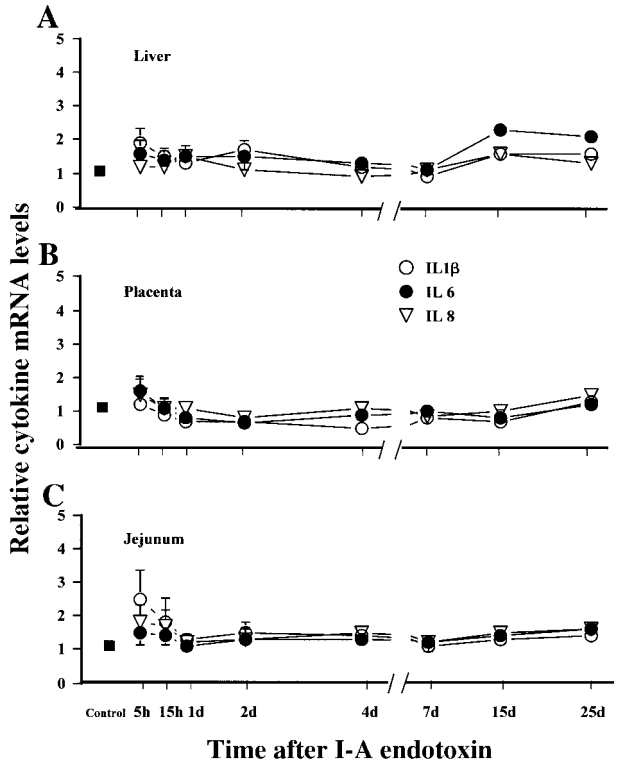

Fig. 7. Intra-amniotic endotoxin did not induce cytokine mRNAs in liver (*A*), placenta (*B*), and jejunum (*C*): mean levels of cytokine mRNA in control animals was designated as 1 (■), and mean ± SE levels at each time point are expressed relative to controls as in Fig. 2. Endotoxin did not increase cytokine mRNA in these tissues; n = 11–12 animals for control and 4–5 animals for each time point except 15 and 25 days, where n = 2–3.

lung response results from direct aspiration of endotoxin. Although fetal breathing and gasps can move pharyngeal fluid into the large airways, the fetal lung produces about 5 $ml \cdot kg^{-1} \cdot h^{-1}$ of fluid, which results in a large net efflux of fluid from the lungs to the amniotic fluid (34). In other experiments, we found that fetal intramuscular doses of 0.05 mg of endotoxin were lethal. Doses of intra-amniotic endotoxin between 1 and 100 mg result in similar lung maturation responses 7 days after exposure, without evidence of fetal toxicity (26). These dose relationships and the time course of induction of proinflammatory cytokine mRNA in chorion/amnion and lung suggest that chorioamnionitis and/or cytokines produced by amnion/chorion may cause fetal lung maturation. This hypothesis could be tested by evaluating cytokine agonists and antagonists for their ability to cause or interfere with lung development. The possibility of a direct endotoxin effect also exists.

The fetal sheep swallows ~50% of the amniotic fluid each day (34). Therefore, unless endotoxin has an extremely short residence time in amniotic fluid, the fetal gut was exposed to a high dose of endotoxin. There was no cytokine mRNA detected in the jejunum, and we also noted no inflammatory infiltrate or change in structure on histopathology. The gut could be signaling the lung by a noninflammatory mediator. We were surprised to find no increased cytokine levels in placental tissue, but this result corresponds to the recent report demonstrating no increase in cytokines in placentas from humans with chorioamnionitis (30). Inflammation in the umbilical cord was not evaluated in the present study. The lack of any substantial increase in cytokine mRNA in the liver is another indicator of a lack of significant exposure of the systemic circulation to endotoxin. However, the changes in peripheral WBCs suggest a systemic response to intra-amniotic endotoxin exposure.

The end point of early lung maturation following endotoxin is different in several aspects from the maturation induced by antenatal glucocorticoids. Maternal or fetal treatment with betamethasone at 104 days gestation causes no or modest changes in lung function following delivery at 125 days gestation (25). However, intra-amniotic endotoxin given at 100 or 110 days gestation resulted in striking increases in alveolar and total lung Sat PC following delivery at 125 days, demonstrating a potent and longer lasting maturation effect. Betamethasone improves postnatal lung function after preterm delivery in 15–24 h, while the endotoxin effects on lung function were not apparent until 4–7 days after the exposure (24). Endotoxin did not increase fetal cortisol levels in this model (26). Therefore the amnionitis is not simply causing fetal stress, which secondarily results in lung maturation, as proposed for infants or models of fetal growth restriction in sheep (16, 47). The term lung maturation is used here to describe the changes in physiology that result in improved lung function. However, the changes may differ from the normal maturational sequence. Morphometric studies indicate that following either intra-amniotic endotoxin or steroid exposure, the alveolar architecture is altered to contain fewer and larger alveoli (50). Transgenic mice overexpressing TNF-α (39), transforming growth factor-α (22), or IL-6 (7) in the lungs also have fewer and larger alveoli than normal. However, precocious lung development was not studied in these transgenic mice. Thus, from a developmental perspective, the induced lung maturation caused by different agents is not normal.

Epidemiological studies have demonstrated the association of elevated proinflammatory cytokines in bronchoalveolar lavage fluid with BPD. Compared with control infants not developing BPD, increased IL-6 (4, 32), IL-8 (29, 31, 37), and IL-1β (32) were found in bronchoalveolar lavage fluid in the first 2 wk of life of preterm infants who later developed BPD. Increased levels of IL-1β, IL-6, IL-8, and TNF-α in the amniotic fluid have been correlated with increased incidence of BPD (17, 51). Thus elevated IL-1β, IL-6, and IL-8 either in the amniotic fluid or in bronchoalveolar lavage fluid predict later development of BPD. Our data suggest that antenatal exposure to these proinflammatory cytokines can be associated with lung maturation. These diverse effects of cytokines may in part be due to the complex biology of signal transduction of these cytokines. The developmental context in which these cytokines are expressed may modify their biological effects. For example, IL-1α can upregulate, have no effect, or downregulate the expression of surfactant protein mRNA depending on the gestation of the rabbit lung explant (18).

These experiments provide initial information about how a fetus responds to an intra-amniotic proinflammatory stimulus. One of the goals for future research is to identify which components of this response may be harmful and result in adverse outcomes in preterm infants. Lung injury may result from the fetal lung being primed by a proinflammatory exposure to respond with an excessive and injurious proinflammatory response to ventilation, as has been demonstrated with exposure of the adult rat lung to endotoxin before mechanical ventilation (45). It also may be that beneficial effects result from signaling molecules that are different from those causing injury. Regardless, intra-amniotic endotoxin causes induced lung maturation that is more potent and longer lasting and that occurs earlier in gestation than the fetal lung responses to glucocorticoids.

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