Role of neutrophils in lung vascular injury and edema after premature birth in lambs

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Carlton, David P., Kurt H. Albertine, Soo Chul Cho, Menno Lont, and Richard D. Bland. Role of neutrophils in lung vascular injury and edema after premature birth in lambs. J. Appl. Physiol. 83(4): 1307–1317, 1997.—To investigate the role of neutrophils in the pathogenesis of respiratory distress after premature birth, we assessed the relationship between circulating neutrophil concentration and neutrophil accumulation in the lung, lung lymph and pleural fluid, and extravascular lung water in 10 chronically catheterized preterm lambs (127 ± 1 days gestation) that were mechanically ventilated for 8 h after birth. Circulating neutrophil concentration transiently decreased within 2 h after birth and then returned to prenatal values by 6–8 h. The increase in circulating neutrophil concentration was related directly to the accumulation of neutrophils in the air spaces, drainage of liquid and protein from the lung 6–8 h after delivery, and postmortem extravascular lung water. In additional studies, we intravenously administered mechlorethamine to 5 fetal lambs to reduce circulating neutrophils before delivery (neutrophil concentration before birth: 9 ± 11 cells/μl). Compared with control lambs, neutrophil-depleted lambs had significantly less drainage of liquid (7.8 ± 5.9 vs. 2.6 ± 1.9 ml/h, respectively) and protein (116 ± 74 vs. 42 ± 27 mg/h, respectively) from the lung 6–8 h after birth and significantly less extravascular lung water at postmortem (6.5 ± 0.8 vs. 4.8 ± 0.6 g/dl lung, respectively). Thus neutrophils contribute to the pathogenesis of respiratory distress after premature birth by increasing lung vascular protein permeability and promoting lung edema.

Respiratory Failure after prematurity birth is a result of incomplete lung development and insufficient surfactant material in the distal air spaces. Pulmonary microvascular injury, lung epithelial damage, and pulmonary edema accompany respiratory failure after premature birth and are apparent by 8 h after delivery (7, 24). Coincident with these pathological findings, neutrophils appear in the distal lung. This suggests that inflammation is an additional component of the acute respiratory distress of prematurity (23).

Evidence for the importance of neutrophils in lung disease derives from the observation that leukopenia frequently precedes the onset of adult respiratory distress syndrome (ARDS) and that neutrophils are abundant in the lungs of patients with this condition (35, 36). In addition, neutrophils from patients with ARDS are altered functionally, releasing excessive amounts of oxidants in response to opsonized zymosan or phorbol myristate acetate (40). One important characteristic of ARDS is an increase in pulmonary vascular permeability, an abnormality that may be linked to neutrophils. In experimental animal models in which the pulmonary circulation is damaged intentionally, neutrophil depletion preserves the barrier function of the lung microcirculation (1, 18, 19, 21, 34).

The present study was done to determine whether the presence of neutrophils in the premature lung after birth was related to the physiological abnormalities of the respiratory distress syndrome of prematurity. We studied preterm lambs before and after birth and found that neutrophils transiently disappeared from the systemic circulation during the first 2 h after delivery but that circulating neutrophil concentrations returned to prenatal values by 6 h after birth. The extent to which the circulating neutrophil concentration declined after birth correlated with the degree of neutrophil sequestration in the lung, fluid, and protein leak from the lung, postmortem extravascular lung water, and respiratory support. When we used mechlorethamine to eliminate neutrophils from the circulation before delivery, fluid and protein leak from the lung diminished, extravascular lung water decreased, and respiratory support was reduced.

METHODS

Surgical preparation. Using methods previously described, we surgically prepared 15 mixed-breed fetal lambs at 119–125 days gestation (term is 147 days gestation) with chronic lung lymph fistulas (7). The chronic lung lymph fistula preparation allows for the measurement of net liquid and protein movement into the lung from the pulmonary circulation before and after birth (7, 8). Using this method, we can detect changes in lung vascular protein permeability by assessing pulmonary vascular pressures and liquid and protein drainage from the lung (7–9).

After administering general anesthesia to the ewe, we performed a midline hysterotomy and placed catheters into the abdominal aorta and inferior vena cava of the fetus through an incision in a hindlimb. We then performed a right thoracotomy, placed a catheter in the pleural space and a catheter in the efferent duct of the caudal mediastinal lymph node, and ligated the tail of the node to eliminate nonpulmonary sources of lymph. In fetal lambs, the caudal mediastinal node receives one-half to two-thirds of total lung lymph (22). A small segment of the lymphatic catheter (1–2 cm length, 0.28 mm ID, 0.61 mm OD) was inserted into the efferent duct and then attached to a longer segment of catheter (80 cm long, 0.76 mm ID, 1.27 mm OD) outside the duct. The interior of the lymphatic catheter was treated to prevent clotting (tridodecylmethylammonium chloride-heparin processing; Polysciences, Warrington, PA). We then performed a left thoracotomy and placed catheters into the pulmonary artery, left atrium, and pleural space. A catheter also was secured to the fetal chest for subsequent delivery of antibiotics and measurement of pressure in the amniotic fluid. All catheters were brought through the uterine incision and exteriorized through the ewe’s flank. After surgery, the ewe and fetus received antibiotic...
ics daily. The study was approved by the Committee on Animal Use at the University of Utah.

Experiments. We performed two sets of experiments. In the first set, we measured neutrophil concentrations in the circulation of 10 premature lambs before and after birth, and we related changes in neutrophil concentration to neutrophil accumulation in the air spaces, drainage of liquid and protein from the lung, postmortem extravascular lung water, and ventilator support. We assessed neutrophil activation in the lung by immunohistochemical staining of tissue sections for CD18 expression.

In a second set of experiments, we administered mechlor- ethamine intravenously (iv) to eliminate neutrophils (Merck, West Point, PA; 2–3 injections, 1–5 mg/injection, average dose 3.1 ± 1.6 mg) beginning 1–6 days postoperatively to five premature fetal lambs before birth. We adjusted the dose and frequency of administration of mechlor ethamine to minimize the prenatal neutrophil concentration in peripheral blood. We studied lung fluid balance before and after birth in the mechlor ethamine-treated lambs and compared the results with those in the 10 control lambs in the first set of experiments. The first eight control lambs served as controls in a study of surfactant replacement at birth that was performed during the same time period (14).

We began the experiments 3–12 days after surgery. We studied the lambs for a 2- to 4-h baseline period before birth and for 8 h after birth. During the study, we continuously measured systemic and pulmonary vascular pressures, pleural pressure, amniotic pressure (before birth), and airway pressure (after birth), and we recorded the averaged values every 15–30 min. We collected lung lymph every 30 min and measured total leukocyte count with a hemocytometer and identified neutrophils by differential staining (Sure Stain; Fisher Diagnostics, Pittsburgh, PA). We measured only segmented neutrophils; band forms accounted for <5% of total neutrophils before and after birth.

We centrifuged samples of blood, lymph, and pleural liquid and measured the concentration of total protein and albumin in the supernatant (10). We measured the pH, PAO2, and PACO2 in samples of arterial blood by using a blood-gas analyzer (model 178; Ciba Corning, Medfield, MA) that was adjusted according to the prevailing body temperature.

We measured total leukocyte count with a hemocytometer and identified neutrophils by differential staining (Sure Stain; Fisher Diagnostics, Pittsburgh, PA). We measured only segmented neutrophils; band forms accounted for <5% of total neutrophils before and after birth.

We centrifuged samples of fetal tracheal fluid at 250 g for 15 min to remove cell debris and then froze the supernatant at −80°C. We extracted the lipids from the supernatant (10) and measured lipid-associated phosphate as an index of surfactant concentration in the lung (3).

Microscopy. To determine the number and distribution of neutrophils in the lung, we placed the right middle lobe or the lingula of the left lobe in 10% neutral formalin and embedded it in paraffin. We then cut sections from the largest cross section of the tissue block for analysis. After staining the tissue with Giemsa or hematoxylin and eosin, we projected the tissue from the light microscope to a high-resolution, calibrated video monitor; the tissue field was overlaid by a computer-generated, coherent square lattice (Bioquant advanced image-analysis system; R&MBiometrics, Nashville, TN). Point-intersection counts were made for lung air spaces, tissue spaces, and neutrophils. We did not include airways in our assessment of air spaces. Neither did we include vascular structures, pleura, or septa in our assessment of tissue space. We counted staggered fields until at least 100 neutrophils were counted (2).

As an indication of neutrophil activation, we qualitatively assessed the expression of CD18 associated with the neutrophils by immunostaining lung sections from three lambs with a monoclonal antibody (R15.7) raised against human CD18 (generously donated by Dr. Robert Rothlein, Boehringer-Ingelheim, Ridgefield, CT) (4, 5). Briefly, the tissue sections were submersed in citrate solution (Citra solution; BioGenex, San Ramon, CA) and treated for 15 min in a microwave oven (600-W magnetron) before being incubated with the primary
antibody (1:5 to 1:50 dilutions) in phosphate-buffered saline. We incubated tissue sections with a biotinylated immunoglobulin G (secondary antibody), followed by incubation with an avidin-peroxidase complex to reveal antibody binding (Vector Elite kit; Vector Laboratories, Burlingame, CA). Gill’s no. 3 hematoxylin was used as the counterstain. Endogenous peroxidase staining was eliminated by treating the tissue sections with 3% hydrogen peroxide in methanol. Our control procedure consisted of immunostaining tissue sections in the absence of the primary or secondary antibody. Control and experimental tissue sections were processed in parallel.

Statistical analysis. Results in the text and tables are expressed as means ± SD. When comparing sample means of one group before and after birth, we used analysis of variance and Dunnett’s test. When comparing sample means between the two groups of lambs, we used an unpaired t-test or the Mann-Whitney test. We used least-squares linear regression to display the relationship between continuous variables. For statistical analysis, we used a commercially available computer program (StatView SE+ Graphics; Abacus Concepts, Berkeley, CA) and standard statistical tables (39). We considered values significantly different if P was < 0.05.

RESULTS

Study 1: Circulating neutrophils and lung fluid balance. Before birth, all 10 lambs were healthy, as assessed by pH, blood-gas tensions, and hematocrit (pH 7.37 ± 0.04; PaO2, 20 ± 2 Torr; PaCO2, 46 ± 3 Torr; hematocrit, 36 ± 4%). The average gestational age was 127 ± 1 days, and birth weight was 3.1 ± 0.6 kg.

Compared with prenatal values, the concentrations of circulating neutrophils in the 10 lambs decreased by between 10 and 95% during the 30–90 min after delivery (Fig. 1). By 8 h after birth, the systemic neutrophil count returned to the prenatal concentration.

The decline in neutrophil concentration 30–90 min after birth was not related to lung liquid or protein drainage during the first 2 h after delivery. However, we found a close relationship between early neutropenia and subsequent drainage of liquid and protein from the lung during the last 2 h of study, 6–8 h after birth (Fig. 2). The disappearance of neutrophils from the circulation also correlated with the amount of extravascular water that was measured in the lungs after death.

The change in circulating neutrophil concentration within 2 h after birth was related also to the degree of respiratory difficulty during the last 2 h of study (Fig. 3). The decrease in neutrophil concentration 30–90 min after birth was related directly to the peak inflation pressure and PaCO2 6–8 h after birth. Mean airway pressure during the final 2 h of study was also related to the decrease in neutrophil concentration 30–90 min after birth (r = 0.82, P < 0.004, data not shown).
found no consistent relationship between the alveolar-arterial O2 difference and the disappearance of neutrophils from the circulation.

Transient neutropenia after birth was associated with subsequent neutrophil sequestration in the lung. The average decrease in neutrophils 30–90 min after delivery correlated directly with the degree of neutrophil accumulation in the distal air spaces at postmortem (Fig. 4). Drainage of liquid and protein from the lung during the last 2 h of study and postmortem extravascular lung water were related to the accumulation of neutrophils in the air spaces (liquid drainage, $r = 0.80$, $P < 0.006$; protein drainage, $r = 0.75$, $P < 0.02$; lung water, $r = 0.74$, $P < 0.02$, data not shown).

To exclude the possibility that atelectasis of the tissue sections influenced our assessment of neutrophil accumulation, we measured the surface area of the tissue section used for analysis, but we found it had no relationship to the neutrophil concentration in the air spaces ($r = 0.31$, $P > 0.40$). Similarly, we measured the average surface area of 100 air spaces in each of four lambs. Two of these lambs had relatively few neutrophils in the lung (and less-severe respiratory distress), and two had a relative abundance of neutrophils in the air spaces (and more-severe respiratory distress). The air space diameters of both groups were not substantially different in size, averaging 43 ± 3 and 37 ± 3 µm, respectively.

Neutrophil concentration in the tissue spaces of the lung (interstitium and microvasculature) was related directly to neutrophil concentration in the distal air space ($r = 0.76$, $P < 0.01$, data not shown). Total neutrophil concentration in the lung (the sum of neutrophils in the air spaces and in the tissue) was related directly to drainage of liquid ($r = 0.83$, $P < 0.004$) and protein ($r = 0.82$, $P < 0.004$) from the lung.

Neutrophils in the air spaces of the lungs, but not those in the lumen of pulmonary arterial vessels, were immunoreactive for CD18 (Fig. 5). In addition, neutrophils in the air spaces and interstitium appeared larger than those remaining in the circulation. Control sections showed no immunostaining in the absence of the primary or secondary antibody.

Neither the decrease in neutrophil concentration soon after birth nor the extent of neutrophil sequestration in the lungs was related to vascular pressure in the pulmonary artery or left atrium or protein concentration in lymph or plasma during the final 2 h of study.

There was no association between mononuclear cells or platelets and lung fluid balance. Although the concentration of mononuclear cells in the lung declined 30–90 min after birth (1,715 ± 824 vs. 1,095 ± 502 cells/µl of blood before delivery vs. 30–90 min after delivery, respectively), the change in circulating mononuclear cells 30–90 min after delivery was not related to drainage of liquid or protein during the last 2 h of study or to extravascular lung water ($P > 0.75$ for any relationship). We measured circulating platelet counts in 5 of the 10 lambs and found no significant change in platelet concentration before and after birth (517,000 ± 133,000 vs. 382,000 ± 102,000 platelets/µl of blood, before delivery vs. 30–90 min after delivery, respectively). The change in platelet concentration 30–90 min after delivery was not related to drainage of liquid or protein during the last 2 h of study or to extravascular lung water ($P > 0.60$ for any relationship).

**Fig. 3.** Relationship between average decline in circulating neutrophil concentration in preterm lambs ($n = 10$ lambs) 30–90 min after delivery and (A) peak inflation pressure ($r = 0.74$, $P < 0.02$) and (B) arterial Pco2 ($PaCO_2$) ($r = 0.74$, $P < 0.02$) during final 2 h of study.

**Fig. 4.** Relationship between average decline in circulating neutrophil concentration in preterm lambs ($n = 10$ lambs) 30–90 min after delivery and subsequent accumulation of neutrophils in lung at postmortem 8 h after delivery ($r = 0.95$, $P < 0.0001$).
Fig. 5. Color photomicrographs showing CD18 immunolocalization in lung tissue from a preterm lamb mechanically ventilated for 8 h after birth. A and B: neutrophils in distal air spaces are stained brown (arrows), indicating CD18 immunoreactivity. C: in contrast, neutrophils free in lumen of pulmonary arteriole (arrows) show no immunoreactivity. Size of neutrophils located in distal air spaces appears larger than that of intravascular neutrophils. D: lack of immunostaining of neutrophils (arrows) in distal air spaces when primary antibody was omitted. A-D are same magnification; scale bar, 10 µm. For comparison, Fig. 7A shows low-power view of lung also mechanically ventilated for 8 h after birth.
Table 1. Net lung liquid flow and protein drainage from lung lymph and pleural space in 15 preterm lambs 2–4 h before birth

<table>
<thead>
<tr>
<th>Group</th>
<th>Net Liquid Drainage, ml/h</th>
<th>Net Protein Drainage, mg/h</th>
<th>Protein Concentration, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph</td>
</tr>
<tr>
<td>Control lambs</td>
<td>2.4 ± 1.2</td>
<td>55 ± 18</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Neutrophil-depleted lambs</td>
<td>2.5 ± 1.4</td>
<td>72 ± 39</td>
<td>2.9 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of lambs.

Table 2. Hemodynamic variables in 15 preterm lambs 2–4 h before birth

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
<td>Pulmonary artery</td>
</tr>
<tr>
<td>Control lambs</td>
<td>45 ± 3</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Neutrophil-depleted lambs</td>
<td>54 ± 7*</td>
<td>55 ± 7*</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of lambs. *Significantly different from control, P < 0.05.

Study 2: Neutrophil-depletion studies. Before birth, pH, blood-gas tensions and hematocrit were normal in the five lambs that received mechlorethamine (pH 7.35 ± 0.04; PaO_2, 19 ± 3 Torr; PaCO_2, 44 ± 4 Torr; hematocrit, 31 ± 4%). The average gestational age was 129 ± 1 days, and average birth weight was 2.9 ± 0.5 kg. Mechlorethamine treatment reduced total leukocyte concentration to a mean of 240 ± 346 cells/µl blood before birth (range, 53–857 cells/µl blood) and neutrophil concentration to a mean of 9 ± 11 cells/µl blood before birth (range, 0–30 cells/µl blood). Leukocyte and neutrophil concentrations remained low during the 8 h of postnatal study (leukocytes, 11–550 cells/µl blood; neutrophils, 0–20 cells/µl blood).

Before birth, net lung liquid and protein drainage (the sum of liquid or protein drainage from lung lymph and pleural space) were similar in the control and neutrophil-depleted lambs (Table 1). Similarly, the concentration of protein in liquid from lymph, pleural spaces, and plasma was similar between the two groups before birth. Aortic and pulmonary arterial pressures were greater in neutrophil-depleted lambs compared with control lambs (Table 2).

During the first 2 h after delivery, drainage of liquid (5.9 ± 3.1 vs. 4.9 ± 2.2 ml/h, control lambs vs. neutrophil-depleted lambs, respectively) and protein (102 ± 41 vs. 105 ± 38 mg/h, control lambs vs. neutrophil-depleted lambs, respectively) from the lung were similar between control lambs and mechlorethamine-treated lambs. Vascular pressure in the aorta, vena cava, and left atrium was no different between the two groups. However, pulmonary arterial pressure was greater in neutrophil-depleted lambs (38 ± 8 vs. 30 ± 9 mmHg, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.02). Peak inflation (41 ± 7 vs. 22 ± 6 cmH_2O, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.0005) and mean airway pressures (12 ± 2 vs. 8 ± 1 cmH_2O, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.0005) were lower in neutrophil-depleted lambs. End-expiratory and pleural pressures were similar in the two groups.

During the final 2 h of study, fluid and protein drainage from the lung was less in the neutrophil-depleted lambs compared with control lambs (Table 3). Lung liquid drainage increased on average from prenatal values by >300% in control lambs, whereas liquid drainage was unchanged in neutrophil-depleted lambs. Protein drainage increased from prenatal values by an average of two- to threefold in control lambs, whereas protein drainage fell or remained constant over the same period in neutrophil-depleted lambs. Although protein concentration in the plasma was greater in neutrophil-depleted lambs during the last 2 h of study, protein concentration in plasma decreased to the same extent in both groups of lambs after birth (22 ± 12 vs. 14 ± 6%, control lambs vs. neutrophil-depleted lambs, respectively). The concentration of protein in lymph was greater in control lambs compared with neutrophil-depleted lambs during the last 2 h of study (0.60 ± 0.08 vs. 0.51 ± 0.08, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.04).

Postmortem extravascular lung water was less in lambs depleted of neutrophils (6.5 ± 0.8 vs. 4.8 ± 0.6 g/dl dry weight of lung, control lambs vs. neutrophil-depleted lambs, respectively; Fig. 6).

Systemic and pulmonary vascular pressures were similar in control and neutrophil-depleted lambs during the last 2 h of study (Table 4). Peak and mean inflation pressures were less in neutrophil-depleted lambs than in control lambs. End-expiratory and pleural pressures were similar for the two groups. In neutrophil-depleted lambs, PaO_2 was higher (90 ± 123 vs. 299 ± 115 Torr, control vs. neutrophil-depleted lambs, respectively; P < 0.007) and PaCO_2 was lower (46 ± 12 vs. 34 ± 5 Torr, control vs. neutrophil-depleted lambs, respectively; P < 0.03). Arterial pH averaged 7.27 ± 0.13 in the control lambs vs. 7.38 ± 0.05 in neutrophil-depleted lambs (difference not significant). Final hematocrit was less in neutrophil-depleted lambs (34 ± 6%) compared with controls (39 ± 4%; P < 0.05).

Control lambs had alveolar edema and hyaline membranes, findings that were absent in neutrophil-depleted lambs (Fig. 7). Neutrophil-depleted lambs had fewer neutrophils in their distal air spaces compared...
with controls (138 ± 88 vs. 5 ± 2 cells/mm² air space, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.006). Neutrophil-depleted lambs also had fewer neutrophils in the tissue space of the lung (interstitium and microvasculature) compared with controls (599 ± 218 vs. 35 ± 7 cells/mm² tissue, control lambs vs. neutrophil-depleted lambs, respectively; P < 0.0002). There were few or no mononuclear cells or immature neutrophils (bands) in the air spaces of the lungs from either control or mechlorethamine-treated lambs.

Mechlorethamine did not accelerate biochemical or structural lung maturation. Lipid-associated phosphate concentration in fetal tracheal fluid was appropriately for this gestational age (20) and similar between the two groups (0.1 ± 0.1 vs. 0.2± 0.2 µg/ml tracheal liquid control lambs vs. mechlorethamine-treated lambs, respectively). Mechlorethamine did not cause thinning of the distal air space wall or formation of secondary alveolar septae (Fig. 7).

**DISCUSSION**

Pulmonary edema is a consistent feature of the respiratory distress syndrome of prematurity. Our results provide new evidence that the disturbance in lung water balance after premature birth may be, in part, a result of neutrophil-mediated lung injury. In prematurely delivered lambs, we found that circulating neutrophils transiently decreased soon after birth and that the decline in circulating neutrophils was associated with the subsequent accumulation of neutrophils in the air spaces. Neutrophils that migrated into the lung, but not those remaining in the circulation, expressed CD18. The temporary decrease in peripheral blood neutrophil concentration after birth and the extent of neutrophil sequestration in the air spaces were related directly to transvascular movement of liquid and protein out of the pulmonary circulation into the lung, extravascular lung water, and respiratory support. In additional experiments, we eliminated neutrophils from the circulation before birth by administration of mechlorethamine. We found that drainage of liquid and protein from the lung after birth decreased, extravascular lung water declined, and gas exchange improved. Thus neutrophils contribute to the pathogenesis of respiratory distress after premature birth by increasing transvascular fluid and protein movement into the lung and promoting formation of pulmonary edema.

A number of variables, including filtration pressure and vascular barrier permeability, regulate transvascular fluid flux and edema formation in the lung. As noted in other studies of granulocyte-mediated lung injury (21), we found that neutrophils had no apparent effect on pulmonary vascular pressures or protein concentrations in lymph and plasma, variables that might be expected to alter transvascular fluid and protein movement. We found no significant difference in pulmonary arterial or left atrial pressures by 6-8 h after birth between control and mechlorethamine-treated lambs, and protein concentrations in lymph and plasma fell to the same degree in both groups by 6-8 h after delivery.

**Table 3. Net liquid flow and protein drainage from lung lymph and pleural space in 15 preterm lambs during last 2 h of study**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Liquid Drainage, ml/h</th>
<th>Protein Drainage, mg/h</th>
<th>Protein Concentration, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymph</td>
</tr>
<tr>
<td>Control lambs</td>
<td>10</td>
<td>7.8 ± 5.9</td>
<td>116 ± 74</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Neutrophil-depleted lambs</td>
<td>5</td>
<td>2.6 ± 1.9*</td>
<td>42 ± 27*</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of lambs. *Significantly different from control, P < 0.05.

**Table 4. Hemodynamic and respiratory variables in 15 preterm lambs during the last 2 h of study**

<table>
<thead>
<tr>
<th></th>
<th>Control Lambs (n = 10)</th>
<th>Neutrophil-Depleted Lambs (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular pressures, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td>56 ± 6</td>
<td>64 ± 8</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>50 ± 14</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Left atrium</td>
<td>3 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Vena cava</td>
<td>5 ± 2</td>
<td>3 ± 1*</td>
</tr>
<tr>
<td>Heart rate</td>
<td>187 ± 28</td>
<td>195 ± 19</td>
</tr>
<tr>
<td>Intrathoracic pressures, cmH2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak inflation</td>
<td>45 ± 13</td>
<td>18 ± 5*</td>
</tr>
<tr>
<td>End expiratory</td>
<td>6 ± 1</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>Mean airway</td>
<td>13 ± 4</td>
<td>8 ± 1*</td>
</tr>
<tr>
<td>Pleural</td>
<td>0 ± 1</td>
<td>1 ± 2</td>
</tr>
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</table>

Values are means ± SD. n, No. of lambs. *Significantly different from control, P < 0.05.
In the control lambs, neither the early postnatal decline in circulating neutrophil concentration nor the extent to which neutrophils sequestered in the lung was related to pulmonary arterial or left atrial pressure or protein concentrations in lymph or plasma. Thus neutrophils do not increase transvascular fluid flux primarily by altering filtration forces in the pulmonary circulation.

Fig. 7. Photomicrographs of lung tissue from 3 preterm lambs. A and B show edema and hyaline membranes (HM) in air spaces and interstitium and neutrophils (N) in distal air space (B, inset) in a preterm control lamb ventilated for 8 h after birth. C and D demonstrate thick air space walls (arrow in D) and mesenchymal hypercellularity in a preterm lamb treated with mechlorethamine before delivery. E and F are from a gestation-matched control fetus that was not allowed to breathe and was not treated with mechlorethamine. Its air space walls (arrow in F) are thick and hypercellular, as expected in a preterm fetal lamb (125 days gestation). We compared lungs from mechlorethamine-treated lambs to lungs from 2 fetal lambs (125 days gestation each) for histology because we found that the presence of edema and atelectasis in control lambs ventilated for 8 h after birth made it difficult to evaluate alveolar development. TB, terminal bronchiole. For purposes of comparison, A, C, and E are photographed at the same magnification (scale bar, 100 µm) as are B, D, and F (scale bar, 10 µm).
More likely, neutrophils increased transvascular fluid movement by increasing lung vascular protein permeability. The greater L/P in control lambs at 6–8 h after birth indicates that the vascular endothelium in this group was more leaky to protein compared with the mechlorethamine-treated lambs. Consistent with this interpretation, protein drainage from the lung in control lambs by 6–8 h after birth was two- to threefold greater than it was in the mechlorethamine-treated lambs. Even within the control group, the effect of neutrophils was apparent. The decline in neutrophil concentration in the circulation during the first 2 h after birth and the extent to which neutrophils accumulated in the lung were related directly to the rate of protein drainage 6–8 h after birth.

Does the change in lung vascular permeability occur before or after neutrophils disappear from the circulation? The results of previous studies argue against an abnormality in the permeability of the microcirculation in the immature sheep lung before or during the time when neutrophils disappear from the peripheral circulation. Lung vascular permeability in premature lambs before birth is similar to that of term lambs, and even in the presence of respiratory distress after premature delivery, the pulmonary microcirculation does not become abnormally leaky within 2 h after birth (7, 15). In the present study, during the initial 2 h after delivery, control and neutropenic lambs had similar ratios of L/P and similar rates of protein drainage from the lung. It was only later, at 6–8 h after birth, that differences in L/P and protein drainage were detectable between the two groups. Similarly, there was no relationship between the early transient neutropenia and the rate of protein drainage within 2 h of birth in control lambs. These findings are most consistent with the notion that lung vascular permeability increases after neutrophils disappear from the circulation.

The interpretation of studies in which neutrophils are removed from the circulation is complicated by the simultaneous depletion of other leukocytes from the blood. However, our results suggest that leukocytes other than neutrophils are not critical to edema formation in this model. In control lambs, we found no relationship between the change in the concentration of mononuclear cells and transvascular protein flux, extravascular lung water, or ventilator support. Moreover, mononuclear cells were absent from the lung. This observation is consistent with that of Jackson et al. (23) who found that in preterm monkeys with respiratory distress, neutrophils accumulated in the lungs within the first day after birth but that other cells, specifically macrophages, did not become abundant in the lungs until 4–5 days after delivery. Thus, although our studies do not exclude a contributory role for mononuclear cells or other components of blood in the pathogenesis of respiratory failure, the neutrophil appears to be the major leukocyte involved.

Our observation of a neutrophil-mediated increase in lung vascular permeability in the respiratory distress syndrome of prematurity is consistent with previous reports demonstrating a harmful effect of neutrophils on the lung microcirculation. The increase in lung vascular permeability produced by a variety of experimental manipulations is inhibited by cyotoxic therapy that reduces circulating neutrophils (1, 18, 21, 34). The mechanism by which neutrophils damage the microcirculation in these studies is not completely clear, but proteolytic enzyme release after degranulation or free radical formation as a result of superoxide generation may contribute to the injury (16).

Activated neutrophils exhibit a spectrum of responses, including expression of CD18, release of intracellular granules, generation of superoxide, and cell enlargement (2, 6, 13, 38). In our experiments, neutrophils in the air spaces appeared to be larger and they expressed qualitatively more CD18 than neutrophils in the pulmonary circulation. This finding suggests that activation had occurred as the neutrophils emigrated from the circulation into the lung. The increase in CD18 immunoreactivity might be a result of an increase in total cellular content of CD18 or only an increase in surface expression; the design of our study does not allow us to distinguish between these two possibilities. A variety of chemoattractants activate neutrophils, but the agonist involved in the stimulation of neutrophils after premature birth is unknown (6, 13).

We cannot exclude completely alternative explanations for the beneficial effect of mechlorethamine on vascular integrity, but it had no effect on the two developmental factors that result in respiratory distress and contribute to lung vascular injury after premature birth: surfactant content and lung maturation (8, 14). We found no difference in lipid-associated phosphate in tracheal fluid, indicating that surfactant content was similar between control and neutrophil-depleted lambs (20). We also evaluated the histological appearance of the lung, but found no evidence that mechlorethamine enhanced the maturation of the lung. The lack of biochemical or anatomical change in the lung after mechlorethamine administration is consistent with the well-recognized genotoxic effect of mechlorethamine (33). It is also unlikely that mechlorethamine reduced lung vascular injury by enhancing endogenous glucocorticoid release. Glucocorticoids administered to the fetus increase the surfactant concentration in the tracheal fluid of fetal sheep, cause the alveolar septae to thin, and initiate secondary septal formation (12, 26, 32). We observed none of these effects in lambs treated with mechlorethamine.

What are the signals that initiate neutrophil sequestration and subsequent migration into the premature lung? Previous studies have shown that high concentrations of inspired O₂ cause neutrophil migration into the newborn lung (28). However, O₂ exposure is an unlikely explanation for our results because neutrophils accumulated in the lungs within 8 h of delivery, a time course inconsistent with the 48–72 h required for O₂-mediated neutrophil recruitment into the newborn lung (28). Moreover, macrophages are the initial inflammatory cell recruited into the lung after excess O₂ exposure (28). We saw few, if any, macrophages in the air spaces by 8 h after birth.

If high concentrations of inspired O₂ are not sufficient to account for neutrophil sequestration and vascular
injury soon after birth, what other factors might be involved? One possibility is that the pattern of lung expansion after premature birth influences neutrophil sequestration and subsequent lung damage. After lavage of the adult lung, a procedure that depletes surface-active material in the air space, lung expansion with conventional tidal ventilation causes neutrophils to sequestrate in the lung, and such accumulation of neutrophils is associated with excess leak of protein into the lumen of the lung (25, 27). However, lung expansion with an alternative form of ventilation, high-frequency oscillation, is not associated with neutrophil accumulation in the air spaces (25, 27). Thus the manner in which the lung is distended may be an important factor that directs neutrophil sequestration in the lung and subsequent injury of the pulmonary microcirculation.

Several investigators have made observations on the role of granulocytes and respiratory distress in premature infants, but these studies focus primarily on the link between neutrophils and the subsequent development of chronic lung disease. During the first week of life, elevated concentrations of neutrophils and elastase in the upper airway are associated with prolonged respiratory support in premature infants (29, 30). Although our study was designed only to study the relationship between neutrophils and the acute lung injury of prematurity, our findings of excess lung water and pulmonary microvascular injury in lambs with neutrophil sequestration in the distal air spaces may help to explain the relationship observed between neutrophils and chronic respiratory disease. If neutrophils found in the upper airway are reflective of previous neutrophil accumulation in the distal air space of the lung, then measuring the change in circulating neutrophil concentration shortly after birth may identify those patients at risk for acute and chronic lung injury. Thus it may be possible to apply strategies soon after birth that will modify neutrophil-mediated lung damage and reduce the severity of acute and chronic respiratory distress.

We thank J. Miciak for technical assistance and S. Marron for preparing the manuscript. This work was supported in part by National Heart, Lung, and Blood Institute Grant HL-40802. Address for reprint requests: D. P. Carlton, Dept. of Pediatrics, Univ. of Utah School of Medicine, 50 North Medical Dr., Salt Lake City, UT 84132.

Received 1 October 1996; accepted in final form 9 June 1997.

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