

# Chronic Lung Injury in Preterm Lambs: Abnormalities of the Pulmonary Circulation and Lung Fluid Balance

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## ABSTRACT

Chronic lung disease of early infancy, or bronchopulmonary dysplasia, is a frequent complication of prolonged mechanical ventilation after premature birth. Pulmonary hypertension and edema are common features of this condition, which is often attributed to long-term, repetitive overinflation of incompletely developed lungs. The overall objective of this work was to examine the effects on the pulmonary circulation and lung fluid balance of different ventilation strategies using large *versus* small inflation volumes in an animal model of bronchopulmonary dysplasia. We studied 16 newborn lambs that were delivered prematurely ( $124 \pm 3$  d gestation, term = 147 d) by cesarean section and mechanically ventilated for 3 to 4 wk. Ten lambs were ventilated at 20 breaths/min, yielding a tidal volume of  $15 \pm 5$  mL/kg, and six lambs were ventilated at 60 breaths/min, yielding a tidal volume of  $6 \pm 2$  mL/kg. All lambs received surfactant at birth and had subsequent surgery for closure of the ductus arteriosus and catheter placement to allow serial measurements of pulmonary vascular resistance and lung lymph flow. Chronic lung injury, documented by serial chest radiographs and postmortem pathologic examination, developed in all lambs irrespective of the pattern of assisted ventilation. Pulmonary vascular resistance, which normally decreases during the month after birth at term, did not change significantly from the first to the last week of study. Lung lymph flow, an index of net transvascular fluid filtration, increased with time in lambs that were ventilated at 20 breaths/min, but not in lambs ventilated at

60 breaths/min. Lymph protein concentration decreased with time, indicative of increased fluid filtration pressure, without evidence of a change in lung vascular protein permeability. Postmortem studies showed interstitial lung edema, increased pulmonary arteriolar smooth muscle and elastin, decreased numbers of small pulmonary arteries and veins, and decreased capillary surface density in distal lung of chronically ventilated lambs compared with control lambs that were killed either 1 d (same postconceptional age) or 3 wk (same postnatal age) after birth at term. Thus, chronic lung injury from prolonged mechanical ventilation after premature birth inhibits the normal postnatal decrease in pulmonary vascular resistance and leads to lung edema from increased fluid filtration pressure. These abnormalities of the pulmonary circulation may contribute to the abnormal respiratory gas exchange that often exists in infants with bronchopulmonary dysplasia. (*Pediatr Res* 48: 64-74, 2000)

### Abbreviations

**BPD**, bronchopulmonary dysplasia  
**CLD**, chronic lung disease of early infancy  
**PVR**, pulmonary vascular resistance  
**Fio<sub>2</sub>**, fraction of O<sub>2</sub> in the inspired gas  
**Pao<sub>2</sub>**, Po<sub>2</sub> in arterial blood  
**Paco<sub>2</sub>**, Pco<sub>2</sub> in arterial blood  
**L/P**, lymph protein concentration to plasma protein concentration

Chronic lung injury often complicates prolonged mechanical ventilation after premature birth, producing a disease that has been called BPD. Three decades after its initial description (1), BPD is the most common cause of long-term hospitalization

and failure to thrive of tiny preterm infants. Although it is generally agreed that this form of chronic respiratory failure reflects abnormal growth and repair of the immature lung exposed to the continuous stress of repetitive inflation with O<sub>2</sub>-enriched gas, the specific cause of this condition remains unclear, and understanding of its pathophysiology is incomplete. These gaps in our knowledge can be attributed, at least in part, to the absence of suitable animal models that include prolonged mechanical ventilation of the immature lung during early postnatal development.

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Most previous experiments designed to produce chronic neonatal lung disease have used mature newborn animals exposed to continuous and prolonged hyperoxia. BPD, however, is unusual after birth at term gestation, and most infants with BPD have not experienced long-term intentional hyperoxia. Thus, one goal of this work was to create an immature animal model of BPD without sustained hyperoxia.

As pulmonary hypertension and lung edema are common features of BPD (2–5), we also set out to see whether long-term mechanical ventilation of prematurely delivered newborn sheep would produce pulmonary vascular abnormalities similar to those observed in preterm human infants with CLD. Because previous studies from our laboratory showed significant increases of both PVR and protein permeability of the lung microcirculation in mature newborn lambs that were mechanically ventilated with high inflation pressures and large tidal volumes for up to 8 h (6), we wondered whether long-term repetitive lung inflation with large tidal volumes and high inflation pressures would have greater effects on the pulmonary circulation and lung fluid balance than would long-term repetitive lung inflation with smaller tidal volumes and lower inflation pressures.

We therefore designed experiments to test the hypothesis that chronic repetitive overinflation of the immature lung, without associated hyperoxia, might lead to pulmonary vascular injury and edema that could be prevented by altering the strategy of assisted ventilation. We delivered fetal sheep prematurely and mechanically ventilated their lungs with either slow, deep inflations or fast, shallow inflations for 3 to 4 wk. Chronic lung injury developed in all lambs irrespective of their pattern of assisted ventilation. Compared with control lambs that were born at term, chronically ventilated preterm lambs had persistent elevation of PVR that was associated with increased lung vascular smooth muscle, reduced numbers of small arteries and veins, diminished capillary surface density, and interstitial pulmonary edema from increased filtration pressure in the lung microcirculation. Although PVR was greater in lambs that were ventilated with slow, deep lung inflations compared with fast, shallow breaths, postmortem abnormalities of the pulmonary circulation were similar in lambs that were managed by either ventilation strategy.

## METHODS

### Preparation of lambs for experiments

**Delivery of lambs.** We used 16 lambs, 12 twins and four singletons, that were delivered prematurely by cesarean section at  $124 \pm 3$  d gestation (range, 120–130 d gestation; term, 147 d). The sheep were mated on a specific day to allow precise knowledge of gestational age. On the day of delivery, the ewes received intramuscular ketamine, 10–20 mg/kg body weight, followed by either spinal anesthesia with 1% tetracaine or inhalational anesthesia with 1% halothane, followed by sterile insertion of catheters into a maternal leg artery and vein. We opened the uterus aseptically through a midline abdominal incision, placed catheters into a neck artery and vein of the fetus, inserted a 4- to 4.5-mm endotracheal tube, withdrew 10 mL of lung liquid, and infused 10 mL of calf lung surfactant

(Infasurf, 35 mg/mL, Ony Inc, Amherst, NY, U.S.A.) into the lung lumen. We then removed the fetus from the uterus, placed the lamb on a neonatal bed beneath a radiant warmer, initiated mechanical ventilation with 100% O<sub>2</sub> via the endotracheal tube, connected the arterial line to a calibrated pressure transducer and electronic recorder to measure blood pressure, and infused a glucose-saline solution (3% glucose, 50 mEq/L NaCl, 25 mEq/L NaHCO<sub>3</sub>) i.v. at an hourly rate of 5–10 mL/kg body weight. The lambs received buprenorphine, 0.03 mg/kg i.v., soon after birth and as needed thereafter to prevent agitation; they also received pancuronium bromide, 0.1 mg/kg i.v., to establish ventilatory control. After delivery, we withdrew heparinized blood from the placenta via the umbilical vein and refrigerated the blood for subsequent transfusions. Uterine, peritoneal, and skin incisions were closed, after which the ewes were kept in a pen and milked at frequently.

**Initial management of the lambs.** The weight of the lambs at birth averaged  $2.55 \pm 0.57$  kg (range, 1.72–3.73 kg). Mechanical ventilation was begun with a time-cycled, pressure-limited infant respirator (Model IV-100B, Sechrist Inc, Anaheim, CA, U.S.A.). The initial respirator rate was 80 breaths/min, inspiratory time, 0.2 sec, end-expiratory pressure, 5 cm H<sub>2</sub>O, and peak-inflation pressure, 30 cm H<sub>2</sub>O, which was adjusted to provide adequate chest rise and maintain PaCO<sub>2</sub> at ~35–45 mm Hg. Initial stabilization of the lambs required up to 24 h, during which time ventilator settings were adjusted to maintain normal PaO<sub>2</sub> and PaCO<sub>2</sub> values. Body temperature was kept between 37° and 38.5°C (normal for sheep) by adjusting the heat output of the overhead radiant warmer. Blood glucose concentrations were monitored with an Exactech glucose measuring device (Medisense Inc, Waltham, MA, U.S.A.), urine output was determined from diaper weights before and after each voiding, and arterial blood was sampled hourly for measurement of pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> on a calibrated blood gas machine (Model 178, Chiron Diagnostics, Norwood, MA, U.S.A.). The lambs received sodium penicillin, 100 mg/kg, i.v. every 12 h, and gentamicin, 2.5 mg/kg every 24 h, for at least 1 wk after birth. If signs and symptoms of sepsis developed thereafter, alternative broad-spectrum antibiotics were given. The lambs received i.v. nutrition with solutions containing glucose, protein, electrolytes, trace metals, and vitamins, and they were fed ewe's milk, as tolerated, through an orogastric tube. The lambs were weighed daily to monitor fluid balance and nutritional status. Serum electrolytes were measured with ion-selective electrodes (Na/K/Cl Stat Analyzer, Model 644, Ciba Corning Diagnostics, Medfield, MA, U.S.A.) as a guide to help adjust fluid and electrolyte management. Chest radiographs were obtained periodically to help assess lung inflation.

During the first several hours after birth, the lambs were kept in a prone position without airway suctioning to avoid removal of the previously administered surfactant. Thereafter, the lambs were rotated from side to side every 4 to 6 h, and their endotracheal tube was flushed with sterile saline solution and suctioned to maintain airway patency. The inspired gas was heated to ~37°C and humidified (InterMed Bear; Bourns Life Systems Div, Riverside, CA, U.S.A.). Respirator tubing was changed three times weekly to reduce the risk of nosocomial infection.

**Surgical preparation.** After the lambs became stable on mechanical ventilation, we performed two thoracotomies, allowing 2 to 3 d for recovery between procedures. The lambs received i.v. fentanyl, 25  $\mu\text{g}/\text{kg}$ , and supplemental doses of 10  $\mu\text{g}/\text{kg}$  if intraoperative tachycardia or hypertension developed. In addition, they received i.v. pancuronium bromide, 0.1 mg/kg, at the start of each procedure. The first thoracotomy was performed within 2 d of birth, at which time we surgically ligated the ductus arteriosus and placed catheters in the pulmonary artery and left atrium. We inserted a 3F thermistor wire (model SP 5003; Gould Inc, Statham Instruments, Oakland, CA, U.S.A.) directly into the pulmonary artery for subsequent measurement of cardiac output by thermodilution (7, 8), and we placed an 8F catheter in the left pleural space for postoperative drainage of air and liquid. After surgery, the lambs received i.v. buprenorphine, 0.01 mg/kg, every 4 to 6 h for analgesia. Two to 3 d later, we performed a right thoracotomy, also with i.v. fentanyl anesthesia. In this procedure, we ligated the tail of the caudal mediastinal lymph node, as previously described, and placed a catheter that was impregnated with heparin (TDMAC Processing; Polysciences Inc, Warrington, PA, U.S.A.) into the efferent duct of this node for collection of nearly pure lung lymph (7, 8). Postoperatively, we administered buprenorphine i.v. to reduce discomfort. We were unable to collect lung lymph successfully from two of the lambs that were mechanically ventilated at 20 breaths/min for 3 wk.

### Experimental protocol

After recovery from surgery, the lambs were maintained on mechanical ventilation at the predetermined respirator rate of either 20 breaths/min or 60 breaths/min. End-expiratory pressure was kept at  $\sim 5$  cm  $\text{H}_2\text{O}$ , peak-inflation pressure was adjusted to maintain  $\text{Paco}_2$  at  $\sim 35$ – $45$  mm Hg, and  $\text{Fio}_2$  was adjusted to keep  $\text{Pao}_2$  at  $\sim 50$ – $90$  mm Hg. The lambs were sedated with either i.v. phenobarbital, 10 mg/kg, or pentobarbital, 3–5 mg/kg, and i.v. buprenorphine, 0.01 mg/kg, as needed. We measured the hematocrit of arterial blood samples by centrifugation daily and gave transfusions of filtered maternal blood if the hematocrit was  $<35\%$ . We gave i.v. infusions of maternal plasma if mean systemic arterial pressure was  $<40$  mm Hg. Daily chest radiographs were taken to assess lung inflation, and white blood cell and neutrophil counts were used to help detect infection.

We measured vascular pressures and cardiac output (measured in triplicate by thermodilution; model SP 1425 Cardiac Output Computer, Gould Inc, Statham Instruments, Oxnard, CA, U.S.A.), lung lymph flow, and protein concentrations in lymph and plasma during steady-state periods of 2–4 h weekly. We measured vascular pressures with calibrated pressure transducers (BT3DC; Statham Instruments) and an eight-channel amplifier-recorder (model 7D; Grass Instruments, Quincy, MA, U.S.A.). We sampled arterial blood hourly for measurement of pH,  $\text{Pao}_2$ ,  $\text{Paco}_2$ , and plasma protein concentration, and we collected lymph continuously in heparin-coated test tubes, with samples taken every 30 min for measurement of volume to the nearest 0.01 mL. Samples of lymph and blood were spun in a centrifuge to obtain supernatant fluids for protein mea-

surements (9). The distal tip of the lymph catheter was kept at a constant height near the level of the left atrium.  $\text{Fio}_2$  was adjusted to maintain  $\text{Pao}_2$  between 50 and 90 mm Hg for 2–4 h of steady-state measurements during studies. We measured tidal volume weekly by electrical integration of gas flow (PEDS Pulmonary Evaluation and Diagnosis System, Medical Associated Services, Hatfield, PA, U.S.A.) through a calibrated pneumotachygraph, as previously described (10).

All 16 lambs included in this report survived for at least 3 wk. After 3 to 4 wk, the lambs received 25 mg/kg i.v. pentobarbital, after which we opened the chest and removed the lungs for subsequent histopathology and measurement of extravascular water.

Seven control lambs that were born at term gestation had surgical ligation of the ductus arteriosus and placement of catheters in the pulmonary artery, left atrium, aorta, and vena cava and a thermistor wire in the pulmonary artery for subsequent weekly studies of PVR. These lambs did not have lymph catheters.

All surgical procedures and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Utah School of Medicine.

### Postmortem studies

We used quantitative methods as previously described (11) to study postmortem lung histopathology. Our analysis focused on the parenchyma and blood vessels of lungs obtained from the 16 chronically ventilated preterm lambs compared with lungs of 10 control lambs that were born at term and killed when they were  $\sim 1$  d old ( $n = 5$ ; weight,  $3.9 \pm 0.7$  kg) or 3 wk old ( $n = 5$ ; weight,  $6.5 \pm 1.9$  kg). Control lambs that were of the same postconceptional age (term,  $\sim 1$  d old) or postnatal age (3 wk old) as the chronically ventilated preterm lambs were mechanically ventilated for 30–60 min at a rate of 30 breaths/min. We also studied lungs of five control fetal lambs that were anesthetized and killed at  $126 \pm 6$  d gestation (weight,  $2.63 \pm 0.68$  kg).

To obtain the lungs for histopathology, we injected heparin (1000 U) and pentobarbital (30 mg/kg body weight) i.v., rapidly split the sternum, double-clamped the lung lobes, and excised the lung tissue with the clamp still attached (11). Lung lobes were clamped at the prevailing peak inflation pressure so that we could relate lung histopathology to the physiologic studies just before death. Clamping the lungs retained air volume, as well as vascular and airspace contents. We placed the clamped right middle lobe in Carnoy's fixative (for quantitative histology), the right cranial lobe in liquid nitrogen, and the left lingula in 10% buffered neutral formalin (for immunohistochemistry). Previous studies indicate that the structure of a specific lung lobe in sheep is similar from animal to animal (12); we therefore used a consistent tissue sampling procedure for each fixation protocol. We used a design-based method to randomly sample the lung (13). Briefly, we cut each piece of lung into 3-mm-thick slabs along parasagittal planes. Large tissue blocks (2–4  $\text{cm}^2$ ; two to three per lamb) were embedded in paraffin and serially sectioned at 5  $\mu\text{m}$  thickness. We used three stains to reveal lung structural features: 1) hematoxylin

and eosin for inflation patterns, edema, and inflammation; 2) Hart's elastic fiber stain for elastin; and 3) Masson's trichrome stain (kit HT15, Sigma Chemical Co., St. Louis, MO, U.S.A.) to differentiate smooth muscle cells from collagen.

Quantitative histology was used to estimate 1) smooth muscle thickness and elastic fiber accumulation in the walls of small pulmonary arteries and veins, 2) number of small pulmonary arteries and veins, and 3) surface density of capillaries in the walls of the distal air spaces. The results reflect two-dimensional area measurements. Three-dimensional estimates were not made because the entire lung was not available for volume displacement or structural analysis. We used a systematic sampling method to evaluate random, nonoverlapping calibrated fields (13) for each variable described below. We used the Bioquant True Color Windows Image Analysis System (R & M Biometrics, Nashville, TN, U.S.A.) to make measurements. Tissue sections were analyzed without knowledge of the lamb group from which the tissue was taken.

To assess abundance of vascular smooth muscle, we traced the external perimeter (vessel area) and the internal perimeter of the media (endothelium plus lumen area). We subtracted the area of the vessel lumen and endothelium from the vessel area to obtain the media area. Results are expressed as the ratio of media area to vessel area. Tissue sections that were processed with Hart's elastic fiber stain were used because elastic fibers delineated the outside and inside boundaries of the vascular smooth muscle layer. We took several steps to select small pulmonary arteries that were of comparable generation, shape and size. First, we used terminal bronchioles as the independent landmark for selecting small pulmonary arteries. Second, only circular (cross-sectional) profiles of the selected small pulmonary arteries were analyzed. Circular profiles were defined as having an  $X$ - $Y$  projection length ratio between 0.8 and 1.2 (a circle has a ratio of 1.0). The  $X$ - $Y$  projection lengths (diameters) and ratio were calculated automatically from the external perimeter tracing of each vessel. Small pulmonary arteries with folded endothelium were excluded to minimize the effect of vessel narrowing on area measurements. Third, the external diameter measurements ( $X$ - $Y$  projection lengths) of the arteries allowed us to verify that the vessels were of similar size within and between lung specimens from different animals. We used the same methods that are described above to estimate smooth muscle abundance around small pulmonary veins. The latter were identified by their location along the perimeter of terminal respiratory units, mostly in the interlobular connective tissue septa. An average of five circular profiles of small arteries and veins were analyzed per lamb.

We determined the area occupied by elastic fibers in the walls of small pulmonary arteries and veins for the same vessel profiles that were used to estimate smooth muscle thickness. For the analysis of elastic fibers, we used automated video threshold analysis of Hart's elastic fiber stain color (11). Briefly, separate color thresholds were set for elastic fibers (stained purple/black) and vascular smooth muscle (stained yellow). The calibrated pixel area for elastic fibers was divided by the calibrated pixel area for vessel wall to calculate percentage area occupied by elastic fibers. An average of five

circular profiles of small pulmonary arteries and veins were analyzed per lamb.

We used two morphometric approaches to estimate the number of precapillary and postcapillary blood vessels per unit area of lung tissue. For the first analysis, blood vessels that were 20–100  $\mu\text{m}$  in external diameter were counted at a video-projected, color image magnification of  $\times 136$ . We did not distinguish between arteries and veins in this analysis because our goal was to determine whether lung blood vessels with diameters ranging from 20 to 100  $\mu\text{m}$  differed in number in the chronically ventilated preterm lambs compared with control lambs. The number of vessels in 15 nonoverlapping, calibrated fields was counted in one random tissue section per lamb. The reference space for the vessel counts was the surrounding lung parenchyma, the area of which was estimated by point-counting, using a 192-point coherent square lattice (11). Using this reference space minimized the effect of different levels of lung inflation because the air space volume density was excluded. For the second analysis, the number of "corner" microvessels was counted at a video-projected, color image magnification of  $\times 347$ . Corner microvessels (15–20  $\mu\text{m}$  in external diameter) were located at the junction of three walls of distal air spaces. The goal of this analysis was to determine whether precapillary and postcapillary microvessels at the junctions of distal air space walls differed in number in the chronically ventilated preterm lambs compared with control lambs. We used the same analytical approach as described above for small arteries and veins.

We used immunohistochemistry and morphometry to estimate the surface density ( $S_v$ ) of capillaries in the walls of distal air spaces. The goal was to determine whether capillary surface area was different in the chronically ventilated preterm lambs compared with control lambs. Endothelial cells of capillaries were highlighted by immunostaining with anti-bovine PECAM-1 antibody (platelet-endothelial cell adhesion molecule-1) (generous gift of Dr. Steven Albelda, University of Pennsylvania, Philadelphia, PA, U.S.A.) and immunoperoxidase stain (14). Color images (magnification  $\times 2860$ ) of 15 to 20 random fields of distal lung tissue per lamb were video-projected onto a cycloid grid [178 points ( $P_T$ ); 2.0 cm cycloid length ( $D_{grid}$ )] for intersection and point counting (13). Intersections ( $I$ ) were counted for the immunoperoxidase-stained endothelium of capillaries ( $I_{cap}$ ). We also counted intersections at the air-tissue interface to determine epithelial surface density ( $I_{epi}$ ). Points overlying lung tissue ( $P_{lu}$ ) were counted for volume density ( $V_v$ ) of lung parenchyma ( $V_v lu$ ). Surface density was calculated from the formula,  $S_v = 2 \times \Sigma I / V_v lu$ , where  $V_v lu = \Sigma P_{lu} \times (D_{grid} / \text{Magnification})$  (13).

### Statistical analysis

Data in the text, tables, and figures are expressed as mean  $\pm$  SD. We used repeated-measures ANOVA to compare physiologic variables that were measured sequentially over time in the two groups of chronically ventilated preterm lambs (15). We used one-way ANOVA and the Student-Newman-Keuls multiple comparison test to identify statistical differences in postmortem measurements among the two groups of chronically

cally ventilated lambs and the three groups of control lambs (16). A  $p \leq 0.05$  defined significant differences.

## RESULTS

Table 1 shows descriptive data for the two groups of chronically ventilated preterm lambs. Gestational age and weights at birth and death were similar, irrespective of ventilation pattern. Likewise, the magnitude of the weight gain that occurred between birth and death was similar for the two groups of lambs. Two lambs that were ventilated at 20 breaths/min were killed at age 28 and 30 d; the other lambs were killed at either 21 d or 22 d after birth.

Because all of the lambs required initial stabilization followed by two surgical procedures during the first week after birth, the wk 1 time delineated the start of the specific ventilation strategy. To assess the effects of ventilation strategy on physiologic variables, we compared data obtained at the end of wk 1 with data obtained at the end of wk 3, at which time we resected the lungs of all but two lambs (those killed at 28 d and 30 d after birth) for histopathology.

Table 2 is a summary of ventilation variables measured at the end of wk 1 and 3 for the two groups of lambs. As expected, tidal volume, peak inflation and mean airway pressures were significantly greater for lambs that were ventilated at 20 breaths/min than for lambs that were ventilated at 60 breaths/min. There were no significant differences for  $\text{FiO}_2$ ,  $\text{PaO}_2$ ,  $\text{Paco}_2$  or arterial blood pH between the two groups of lambs. Likewise, end-expiratory pressure (5–6 cm  $\text{H}_2\text{O}$ , data not shown) was similar for the two groups of lambs.

Table 3 lists pulmonary hemodynamic data for the two groups of lambs. There were no significant changes with time for pulmonary arterial or left atrial pressures or cardiac output for either group of lambs. Likewise, PVR did not change significantly between wk 1 and 3. When expressed per kilogram body weight, PVR was less in lambs that were ventilated at a rate of 60 breaths/min versus 20 breaths/min (Fig. 1). PVR at the end of wk 3 was significantly greater for both groups of chronically ventilated preterm lambs than it was for 3-wk-old term lambs (Table 3).

Lung lymph flow consistently increased between wk 1 and 3 in lambs that were ventilated at 20 breaths/min, whereas there was no significant change in lung lymph flow with time in

**Table 1.** Descriptive data (mean  $\pm$  SD) for the two groups of chronically ventilated preterm lambs

Variable	Group 1 20 breaths/min	Group 2 60 breaths/min
Number of lambs	10	6
Gestation (d)	124 $\pm$ 3	124 $\pm$ 2
Birth weight (kg)	2.69 $\pm$ 0.52	2.32 $\pm$ 0.63
Final weight (kg)	3.13 $\pm$ 0.74*	2.92 $\pm$ 0.86*
Duration of mechanical ventilation	23 $\pm$ 3	21 $\pm$ 0
Male/female	3/7	3/3
Singletons/twins	3/7	1/5

\* Final weight was significantly greater than birth weight for both groups of lambs,  $p < 0.05$ , but neither birth weight nor final weight were significantly different between the two groups of lambs.

**Table 2.** Ventilation data (mean  $\pm$  SD) for the two groups of chronically ventilated preterm lambs

Variable	Group 1 (n = 10) 20 breaths/min		Group 2 (n = 6) 60 breaths/min	
	1 wk	3 wk	1 wk	3 wk
$\text{FiO}_2$	0.42 $\pm$ 0.15	0.46 $\pm$ 0.23	0.49 $\pm$ 0.10	0.43 $\pm$ 0.12
Tidal volume (mL/kg)	11 $\pm$ 3	15 $\pm$ 4	5 $\pm$ 2*	7 $\pm$ 1†
Peak inflation pressure (cm $\text{H}_2\text{O}$ )	29 $\pm$ 8	33 $\pm$ 16	21 $\pm$ 5*	21 $\pm$ 6†
Mean airway pressure (cm $\text{H}_2\text{O}$ )	10 $\pm$ 2	11 $\pm$ 4	8 $\pm$ 2*	8 $\pm$ 2†
$\text{PaO}_2$ (mm Hg)	75 $\pm$ 19	82 $\pm$ 19	73 $\pm$ 13	74 $\pm$ 11
$\text{Paco}_2$ (mm Hg)	37 $\pm$ 3	36 $\pm$ 18	39 $\pm$ 7	33 $\pm$ 4
Arterial blood pH	7.40 $\pm$ 0.05	7.39 $\pm$ 0.09	7.40 $\pm$ 0.04	7.36 $\pm$ 0.04

\* Significant difference between the two groups at the end of wk 1,  $p < 0.05$ .

† Significant difference between the two groups at the end of wk 3,  $p < 0.05$ .

**Table 3.** Pulmonary hemodynamic data (mean  $\pm$  SD) for the two groups of chronically ventilated preterm lambs

Variable	Group 1 (n = 10) 20 breaths/min		Group 2 (n = 6) 60 breaths/min	
	1 wk	3 wk	1 wk	3 wk
Pulmonary arterial pressure (mm Hg)	20 $\pm$ 4	23 $\pm$ 11	21 $\pm$ 2	18 $\pm$ 3
Left atrial pressure (mm Hg)	4 $\pm$ 2	5 $\pm$ 2	7 $\pm$ 1	6 $\pm$ 1
Cardiac output (L/min)	0.91 $\pm$ 0.45	0.98 $\pm$ 0.28	1.08 $\pm$ 0.49	1.09 $\pm$ 0.48
Pulmonary vascular resistance (mm Hg $\cdot$ min $\cdot$ L $^{-1}$ )	23.0 $\pm$ 14.7	20.3 $\pm$ 13.5	14.3 $\pm$ 6.0	13.3 $\pm$ 4.3*

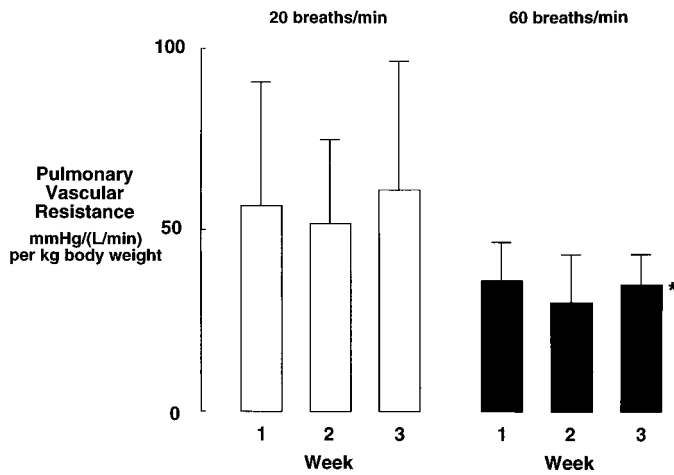
No significant differences between groups or between study wk.

\* For comparison, PVR of seven control term lambs averaged 10.5  $\pm$  2.8 at the end of wk 1 vs 5.8  $\pm$  1.3 at the end of wk 3, a decrease of 43  $\pm$  13%. The difference in PVR between control term lambs and both groups of chronically ventilated preterm lambs at the end of wk 3 was statistically significant,  $p < 0.05$ .

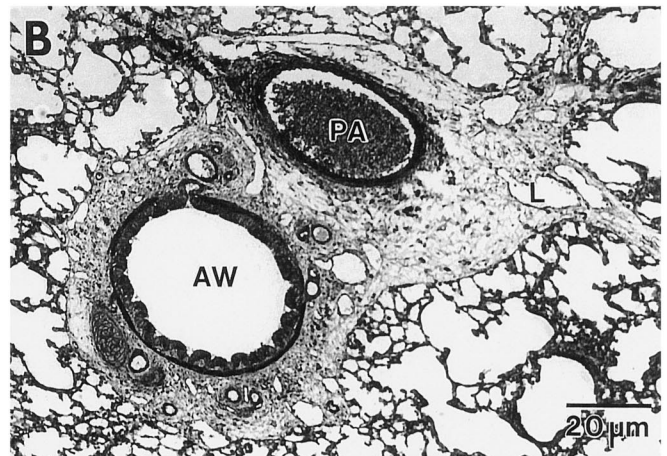
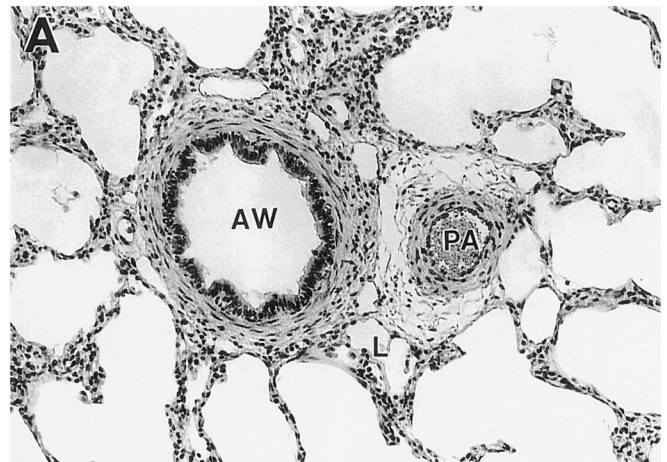
those lambs that were ventilated at 60 breaths/min (Fig. 2). The L/P ratio decreased significantly between wk 1 and 3 in lambs that were ventilated at 20 breaths/min; the decrease in L/P did not reach statistical significance ( $p = 0.1$ ) in lambs that were ventilated at 60 breaths/min (Fig. 3). There was no significant change in lymph protein clearance (lymph flow  $\times$  L/P) for either group of lambs.

Chest radiographs on d 1 showed granular lungs with air bronchograms, consistent with a diagnosis of hyaline membrane disease, progressing to nonuniform inflation and scattered lung opacities, indicative of atelectasis or edema, or both, by the end of wk 3 (not shown). The mix of hyperlucent and dense areas observed at 3 wk was similar to radiographs of infants with typical BPD (1).

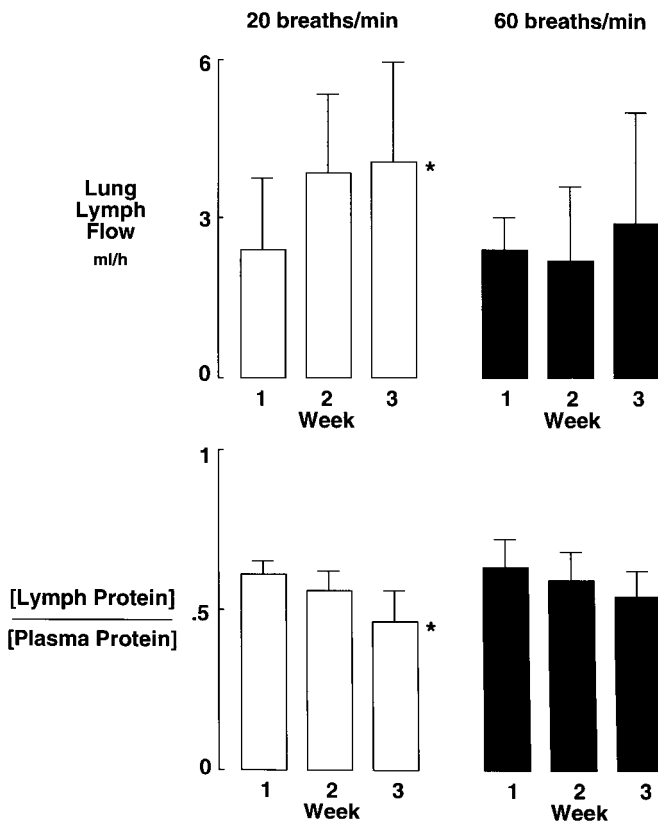
Postmortem measurements of extravascular lung water showed pulmonary edema in both groups of chronically ventilated preterm lambs compared with control lambs. Extravascular lung water tended to be greater in those lambs that were ventilated at 60 breaths/min (6.9  $\pm$  1.5 g/g dry lung tissue) compared with lambs that were ventilated at 20 breaths/min (5.6  $\pm$  1.2 g/g dry lung tissue), but the difference did not reach statistical significance ( $p = 0.09$ ). Extravascular lung water



**Figure 1.** PVR in preterm lambs that were mechanically ventilated for 3 wk at either 20 breaths/min ( $n = 10$ ) or 60 breaths/min ( $n = 6$ ) and studied at weekly intervals. Results are mean and SD; \* indicates significant difference between the two groups of preterm lambs, as assessed by repeated-measures ANOVA,  $p < 0.05$ , without a significant difference between the weekly measurements for either group.



**Figure 3.** Photomicrographs of lung tissue, stained with hematoxylin and eosin, from preterm lambs that were mechanically ventilated at 20 breaths/min (A) or 60 breaths/min (B) for 3 wk. Both lungs show evidence of interstitial edema, including fluid accumulation around blood vessels (pulmonary arteries, PA), airways (AW), within lymphatics (L), and in the interlobular fissures.



**Figure 2.** Steady-state lung lymph flow and L/P (mean  $\pm$  SD) measured at weekly intervals in preterm lambs that were mechanically ventilated at 20 breaths/min ( $n = 8$ ) or 60 breaths/min ( $n = 6$ ) for 3 wk. \* indicates significant difference compared with wk 1,  $p < 0.05$ , as assessed by repeated-measures ANOVA.

was significantly greater in both groups of chronically ventilated preterm lambs than it was in control term lambs that were either 1 d old ( $4.5 \pm 0.4$  g/g dry lung tissue) or 3 wk old ( $4.0 \pm 0.1$  g/g dry lung tissue). Figure 3 shows representative photomicrographs of lung sections from lambs that were ventilated at 20 breaths/min (Fig. 3A) and 60 breaths/min (Fig. 3B),

demonstrating interstitial, but not alveolar, edema. There was considerable variability in the apparent amount of lung interstitial fluid between animals.

It is noteworthy that four lambs, three that were ventilated at 20 breaths/min (only one of which had a lymph catheter) and one that was ventilated at 60 breaths/min, had culture-proven sepsis that was associated with transiently increased ventilatory needs and accentuated pulmonary vascular abnormalities that resolved with appropriate antibiotic therapy. In two such lambs, lung lymph flow increased by an average of 140% and L/P increased from 0.51 to 0.69 during infection. Both lung lymph flow and L/P returned to previous baseline values after successful treatment of the infection. Pulmonary vascular dysfunction, as assessed by measurements of PVR, lung lymph flow, L/P, and extravascular lung water at the end of wk 3, was not significantly different in the four lambs that had documented sepsis from that in the 12 lambs without proven sepsis (data not shown).

Morphometric studies showed that vascular smooth muscle was increased around small pulmonary arterioles, but not around venules, of chronically ventilated preterm lambs compared with control term lambs (Fig. 4 and Table 4). Parallel

studies of elastin fiber density around lung vessels showed significantly greater elastin abundance in both small arteries and veins of chronically ventilated preterm lambs compared with both preterm fetal and term newborn controls (Table 4). In related studies, we also found that mRNA for tropoelastin was significantly increased in preterm lambs that were mechanically ventilated for 3 wk at either 20 or 60 breaths/min (17). This increase in lung tropoelastin mRNA was detectable as early as 3 d after birth in chronically ventilated preterm lambs (18).

Quantitative histology showed fewer small arteries and veins in the lungs of preterm lambs that were mechanically ventilated for 3 wk at either 20 or 60 breaths/min than there were in the lungs of control lambs that were killed either 1 d or 3 wk after birth at term (Fig. 5). This reduction in the number of microvessels (20–100  $\mu\text{m}$  diameter) was approximately half the normal increase in vessel number that occurs during the last 20–25 d of gestation in fetal sheep. There was no significant difference in the average diameter of lung microvessels in chronically ventilated lambs compared with any of the groups of control lambs (data not shown).

Histology of the lung microcirculation also showed reduced capillary surface density in the walls of distal airspaces of preterm lambs that were mechanically ventilated for 3 wk at either 20 or 60 breaths/min compared with the lungs of control lambs that were killed either 1 d or 3 wk after birth at term (Table 5). This diminished capillary surface density is similar to the capillary surface density in fetal lambs at  $\sim 126$  d gestation. Because distal airspaces exhibit a saccular structure in the incompletely developed lungs of premature lambs, in contrast to the alveolar structure in lungs of mature newborn lambs, our study design included parallel assessment of epithelial surface density as a reference index for capillary surface density. We selected for analysis tissue sections that had comparable epithelial surface area for the different groups of lambs. Thus, by design, epithelial surface density was similar between chronically ventilated preterm lambs and control

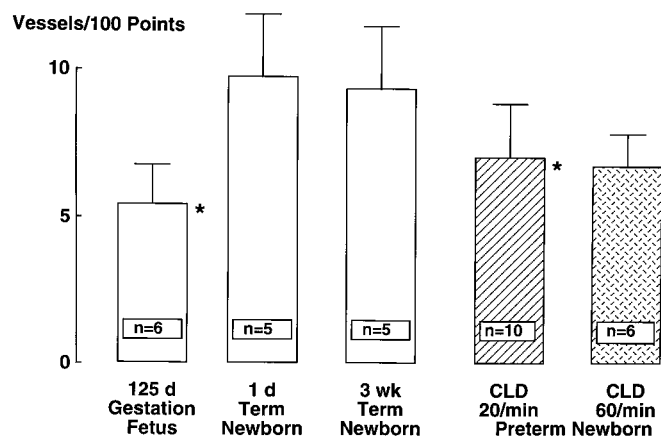
**Table 4.** Smooth muscle abundance and elastic fiber density (mean  $\pm$  SD) in small pulmonary arteries and veins of chronically ventilated preterm lambs and control lambs

Lambs	Vascular Smooth Muscle (media area/vessel area)		Lung Vessel Elastic Fibers (% of smooth muscle media)	
	Arteries*	Veins	Arteries*	Veins
<b>Preterm</b>				
20 breaths/min ( $n = 9$ )	0.43 $\pm$ 0.03 $\ddagger$	0.18 $\pm$ 0.06	15 $\pm$ 3 $\ddagger$	21 $\pm$ 5 $\ddagger$
60 breaths/min ( $n = 5$ )	0.42 $\pm$ 0.11 $\ddagger$	0.15 $\pm$ 0.02	17 $\pm$ 3 $\ddagger$	21 $\pm$ 3 $\ddagger$
<b>Control</b>				
Fetus, 126 d gestation ( $n = 5$ )	0.47 $\pm$ 0.08 $\ddagger$	0.13 $\pm$ 0.02	1 $\pm$ 1	2 $\pm$ 1
Term, 1 d old ( $n = 5$ )	0.27 $\pm$ 0.07	0.14 $\pm$ 0.02	2 $\pm$ 3	2 $\pm$ 2
Term, 3 wk old ( $n = 5$ )	0.22 $\pm$ 0.08	0.14 $\pm$ 0.01	3 $\pm$ 1	5 $\pm$ 2

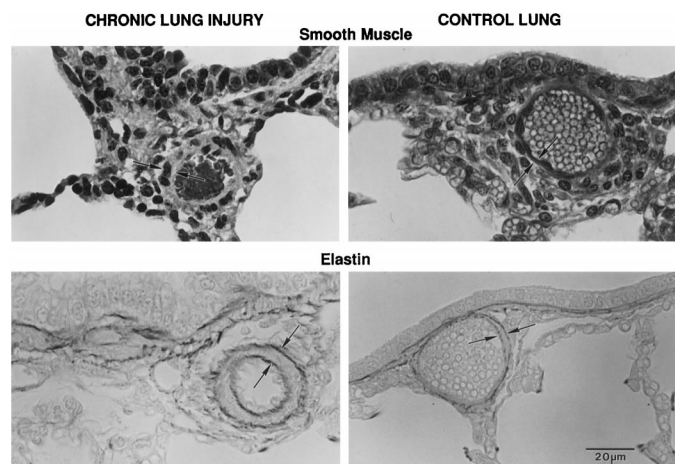
\* Independent landmark for pulmonary arteries was the terminal bronchiole.

$\ddagger$  Significant difference compared with both groups of term control lambs,  $p < 0.05$ .

$\ddagger$  Significant difference compared with all three groups of control lambs,  $p < 0.05$ .



**Figure 5.** Number (mean  $\pm$  SD) of small blood vessels (20–100  $\mu\text{m}$  diameter) per 100 points plotted on a coherent square lattice for sections of lung obtained from chronically ventilated preterm lambs, control fetuses, and newborn lambs that were killed either 1 d or 3 wk after birth at term gestation. \* indicates significant difference compared with 1-d-old and 3-wk-old term newborns,  $p < 0.05$ .



**Figure 4.** Photomicrographs of lung from a chronically ventilated preterm lamb (Left) and a control term lamb (Right) showing smooth muscle thickness (arrows) around small pulmonary arteries (Top, trichrome-stained tissue sections), and elastin fibers surrounding the arterial smooth muscle (Bottom, Hart's elastic fiber stain).

lambs. This is an important consideration, as previous studies showed that alveolar septation and radial alveolar counts are less in the lungs of chronically ventilated preterm lambs compared with control term lambs (11). Therefore, the reduced capillary surface density noted in our lambs with chronic lung injury is not simply a reflection of diminished alveolar surface area.

## DISCUSSION

**Animal model of BPD.** A primary aim of this work was to create an animal model of CLD of early infancy that would mimic the pathophysiology and histopathology observed in human infants with BPD, with particular attention to the pulmonary vascular abnormalities and lung edema that occur in this condition. Because BPD occurs almost exclusively after premature birth and several days or weeks of assisted ventilation with supplemental  $\text{O}_2$ , very few investigators have at-

**Table 5.** Capillary and epithelial surface density (mean  $\pm$  SD) in the walls of distal airspaces in the lungs of preterm lambs that were mechanically ventilated for 3 wk compared with control lambs

Lambs	Surface Density/cm <sup>-1</sup>	
	Capillary	Epithelium
Preterm		
20 breaths/min ( <i>n</i> = 9)	261 $\pm$ 80*	1006 $\pm$ 157
60 breaths/min ( <i>n</i> = 5)	275 $\pm$ 26*	996 $\pm$ 96
Control		
Fetus, 126 d gestation ( <i>n</i> = 5)	177 $\pm$ 45*	760 $\pm$ 236
Term, 1 d old ( <i>n</i> = 5)	401 $\pm$ 122	1027 $\pm$ 240
Term, 3 wk old ( <i>n</i> = 5)	529 $\pm$ 158†	824 $\pm$ 172

\* Significant difference compared with both groups of term control lambs, *p* < 0.05.

† Significant difference compared with term, 1-d-old control lambs, *p* < 0.05.

tempted to reproduce the human disease in a comparable animal model. Escobedo *et al.* (19) and Coalson *et al.* (20–23) developed a model of CLD with the morphologic features of BPD in preterm baboons that were treated with positive-pressure ventilation and prolonged hyperoxia. This group of investigators recently conducted studies of chronically ventilated, extremely premature baboons without associated hyperoxia, but the small size and instability of these animals prohibited invasive studies of the lung circulation (24).

To simulate conditions that usually prevail in the management of human infants who acquire BPD, we elected to keep the inspired O<sub>2</sub> concentration at a level sufficient to maintain normal arterial oxygenation, and we used prematurely delivered lambs of sufficient size to allow surgical placement of vascular and lymphatic catheters for repeated assessment of pulmonary hemodynamic variables and lung fluid filtration as a function of time. Mechanical ventilation of these immature animals for 3 to 4 wk led to chronic lung injury that included many of the pathologic features observed in the lungs of infants with BPD. As previously described (11, 17), the lungs of these chronically ventilated preterm lambs exhibit nonuniform inflation, impaired alveolar formation, abnormal abundance and distribution of elastin in terminal respiratory units, increased muscularization of terminal bronchioles, and inflammation. As reported here, these chronically ventilated preterm lambs also exhibit increased smooth muscle in small pulmonary arteries but not veins, increased abundance of elastin in both small pulmonary arteries and veins, reduced numbers of lung microvessels, diminished surface density of lung capillaries, and interstitial pulmonary edema. These findings are similar to the morphologic abnormalities that have been described in lungs of infants who have died with BPD (1, 2, 25–30).

The physiologic abnormalities that developed in these lambs also are consistent with the limited data that exist from studies of human infants with BPD, notably abnormalities of pulmonary vascular and airway resistance (3, 4, 31, 32). Our observations regarding lung lymph flow and lymph protein concentration in chronically ventilated lambs obviously are not subject to verification in human infants with BPD.

There are at least two notable differences in the conditions that prevailed in our chronically ventilated preterm lambs compared with preterm infants who acquire BPD. First, the

lambs were delivered prematurely without prior labor and without apparent chorioamnionitis; in contrast, most infants in whom BPD develops have been born after the onset of preterm labor, sometimes complicated by the presence of intrauterine infection (33). Second, our lambs all underwent surgical ligation of the ductus arteriosus within a few days of birth, whereas human infants in whom BPD develops sometimes have a patent ductus arteriosus with an associated increase of pulmonary blood flow for several days after birth (33). Despite these differences, the clinical course of our lambs was very similar to that of infants who acquire BPD, and the comparable lung pathology of the chronically ventilated preterm lambs and human infants provides compelling evidence that this animal model should be useful for studying the pathogenesis of BPD and for testing various therapeutic interventions. Our studies also imply that neither intrapartum infection nor prolonged excessive pulmonary blood flow are essential predisposing conditions for the development of CLD after premature birth.

**Pathophysiology of the pulmonary circulation in chronically ventilated preterm lambs.** Another goal of this work was to examine the pathophysiology of the pulmonary circulation during development of CLD in mechanically ventilated preterm lambs. As in babies with BPD who have been studied with cardiac catheterization (3, 4), all of the lambs had persistent elevation of PVR that was associated with increased smooth muscle and elastin around small pulmonary arteries and, in the case of elastin, around small veins as well. Arteriolar smooth muscle mass in the lungs of chronically ventilated lambs was similar to the arteriolar smooth muscle mass in the lungs of fetal lambs that were studied at  $\sim$ 125 d gestation, the same gestational age at which the chronically ventilated lambs were delivered. Thus, the excess lung vascular smooth muscle observed in these animals after 3 wk of mechanical ventilation likely reflects failure of postnatal regression, rather than excess postnatal growth, of vascular smooth muscle.

The increased smooth muscle observed in pulmonary arteries of our chronically ventilated preterm lambs is similar to the smooth muscle overgrowth that occurs in newborn animals exposed to chronic hypoxia (34). Chronic hyperoxia also can lead to pulmonary vascular remodeling, with hypertrophy of arterial smooth muscle and reduced numbers of small pulmonary arteries (35). It is unlikely, however, that either chronic hypoxia or chronic hyperoxia was responsible for the structural abnormalities noted in our preterm lambs, as Pao<sub>2</sub> values <50 mm Hg were extremely uncommon during the course of these studies, and the inspired O<sub>2</sub> concentration that these animals received was generally <60%. Yet, it is impossible to rule out intermittent episodes of hypoxemia or prolonged exposure to supplemental O<sub>2</sub> as contributory variables in the persistence of lung vascular smooth muscle in these studies.

Another possible explanation for this abnormal abundance of arterial smooth muscle is that long-standing mechanical ventilation of immature lungs with supplemental O<sub>2</sub> caused a decrease in the production of nitric oxide, which has been shown to inhibit vascular smooth muscle growth *in vitro* (36, 37). Recent observations in our laboratory showed that the expression of both endothelial nitric oxide synthase and soluble guanylate cyclase are reduced in the lung circulation of our

chronically ventilated preterm lambs (38, 39). These enzymes, which are normally present in the circulation of the developing lung in sheep (40), play an essential role in the production of cGMP and resultant relaxation of vascular smooth muscle. These observations may help to explain why i.v. acetylcholine, an endothelial-dependent vasodilator, did not reduce PVR early in the course of our lambs with evolving CLD, and why inhaled nitric oxide had no apparent effect on PVR in the presence of established CLD after 3 wk of mechanical ventilation (41, 42).

The increased abundance of elastin in small pulmonary arteries and veins of our chronically ventilated preterm lambs could be the result of early postnatal pulmonary inflammation, with associated release of neutrophil elastase causing elastin degradation and new elastin production (29, 43, 44). The discovery that tropoelastin mRNA expression was increased in the lungs of our preterm lambs as early as 3 d after birth is consistent with this notion (17, 18). As maximal lung elastin deposition occurs during alveolar development late in gestation in fetal sheep (45), with a notable increase of elastin in septal crests (46), postnatal lung inflammation after premature birth and mechanical ventilation might be expected to disrupt normal alveolar formation and thereby reduce the number of alveoli (11). Because respiratory unit arteries develop as alveoli multiply (47), impaired alveolar formation would be expected to reduce the number of microvessels that appear in the lung, as noted in our chronically ventilated preterm lambs. Thus, the early postnatal inflammation that occurs in acute lung disease after premature birth likely accounts for at least some of the structural abnormalities of the pulmonary circulation that we observed in our chronically ventilated preterm lambs. It is also possible that repetitive stretching of the lung parenchyma enhances tropoelastin gene expression, yielding increased accumulation of elastin fibers around blood vessels and in distal respiratory units (17, 18). It is noteworthy that lung tropoelastin expression decreases in newborn rats that are exposed continuously to high concentrations of inspired O<sub>2</sub> (48). Thus, it is unlikely that chronic exposure to supplemental O<sub>2</sub> was responsible for the increased abundance of lung elastin in the premature lambs that we studied.

Based on earlier observations that repetitive overinflation of the newborn lung with large tidal volumes leads to consistent abnormalities of lung microvascular protein permeability (6, 49), we expected that sustained mechanical ventilation with large tidal volumes for 3 to 4 wk might lead to more severe lung injury than mechanical ventilation with smaller tidal volumes did. Our results fail to support this hypothesis. Although average PVR was greater in lambs that were ventilated at 20 breaths/min than in lambs that were ventilated at 60 breaths/min, there was not a significant decrease in PVR with time for either group of lambs. Whereas lung lymph flow increased significantly with time, with an associated decrease of L/P, in lambs that were ventilated at 20 breaths/min, there were not significant changes with time for either lymph flow or L/P in lambs that were ventilated at 60 breaths/min. These differences between groups probably do not reflect differences in lung injury, however, as extravascular lung water was not greater in the lambs that received large tidal volumes at a slow

respirator rate. Moreover, the lower L/P that developed in this group of lambs is most consistent with increased lung fluid filtration pressure rather than increased vascular protein permeability (50).

With respect to lung vascular pathology, there were no appreciable differences between lambs that were mechanically ventilated by the two different strategies. The apparent absence of an effect of ventilation pattern on vascular accumulation of elastin contrasts with our previously reported observation that elastic fiber abundance in the walls of distal airspaces was significantly greater in lambs that were mechanically ventilated at 20 breaths/min than it was in lambs that were ventilated at 60 breaths/min (11). The reason for this apparent difference is unclear. It is possible that increased abundance of elastin within terminal respiratory units, as previously reported (11), may have contributed to the greater PVR observed in lambs that were ventilated with slow, deep lung inflations compared with lambs that were ventilated with fast, shallow breaths. We also reported previously that the volume density of alveolar secondary crests was significantly less in lambs that were ventilated at 20 breaths/min compared with those that were ventilated at 60 breaths/min (11). Because capillaries form within alveolar secondary crests, there may have been fewer alveolar capillaries in the lungs of lambs that were ventilated with slow, deep lung inflations than in the lungs of lambs that had more rapid lung inflations. Such a difference in microvascular number would not have been detected in our analysis of capillary surface density normalized to epithelial surface density, and yet it could help to explain the difference in PVR and lymph flow observed in lambs that were managed using the two different ventilation strategies. Consistent with this notion, Coalson *et al.* (24) recently reported a significant reduction in the volume density of vascular endothelium in the lungs of preterm baboons that were mechanically ventilated for several weeks at a respirator rate of 40 breaths/min or less. These observations indicate that long-term mechanical ventilation of the immature lung yields chronic injury to the pulmonary circulation without increasing lung microvascular permeability, and that neither ventilation strategy prevents structural abnormalities of the lung vasculature.

**Pulmonary edema in chronic lung disease.** Pulmonary edema is a consistent pathologic feature of both the acute and chronic lung diseases that occur after premature birth and mechanical ventilation (5). In acute lung disease, or respiratory distress syndrome, edema results from inflammation and an associated increase of pulmonary vascular and epithelial permeability, with protein-rich fluid in both the lung interstitium and airspaces (7, 14, 51). In CLD after premature birth, however, the mechanism responsible for the excess lung fluid is less clearcut. Some investigators have attributed the pulmonary edema that occurs in BPD to abnormal protein permeability (52, 53), whereas other investigators have demonstrated increased filtration pressure in the pulmonary circulation as a likely cause of lung edema in BPD (3, 4, 54).

In this work, we found that long-term repetitive lung inflation with large tidal volumes at a slow respirator rate (20 breaths/min) was associated with a significant increase in lung lymph flow between wk 1 and 3 of mechanical ventilation.

This change in lymph flow was accompanied by a progressive decrease in L/P. These changes are indicative of increased fluid filtration pressure in the pulmonary microcirculation, and they are different from results observed in cases of increased lung vascular permeability, as in bacterial sepsis (55, 56) or pulmonary O<sub>2</sub> toxicity (57), in which L/P either remains constant or increases as lymph flow increases (50). Lung lymph flow at the end of wk 3 was significantly greater in lambs that were mechanically ventilated at 20 breaths/min ( $4.1 \pm 1.9$  mL/h) than it was in prior studies of mature lambs ( $2.6 \pm 0.9$  mL/h) that were 2 to 3 wk old after birth at term (58). Moreover, L/P was significantly less in the preterm lambs ( $0.46 \pm 0.10$ ) than it was in the more mature animals ( $0.62 \pm 0.05$ ) that we studied previously (58). These findings are consistent with a greater lung microvascular filtration pressure in chronically ventilated preterm lambs compared with normal term lambs.

In the six preterm lambs that received prolonged mechanical ventilation at 60 breaths/min, there was not a significant increase in lung lymph flow, and the decrease in L/P between wk 1 and 3 did not reach statistical significance ( $p = 0.1$ ). These differences in physiologic variables, when compared with results of studies performed on lambs that were mechanically ventilated at 20 breaths/min, might be related to the observed differences in PVR between groups, or to the greater degree of atelectasis seen in the lungs of lambs that received more rapid, shallow ventilator breaths (11). Raj (59) showed that interstitial pressure in the immature lung is less in the presence of atelectasis than it is when the lungs are well inflated after administration of surfactant. Because a low lung interstitial pressure is likely to increase transvascular fluid filtration and, at the same time, reduce the driving force for lymphatic drainage of fluid from the lungs, one might expect an association between atelectasis and diminished lymph flow, perhaps accentuating lung edema.

Extravascular lung water was significantly greater in both groups of chronically ventilated preterm lambs than it was in control lambs that were born at term gestation. Lung histology showed that this increase in extravascular lung water was primarily within the interstitial space, with notable cuffs of fluid around large blood vessels and airways, as well as dilated lymphatics and prominent interlobar fissures. Alveolar fluid was unusual and occurred mainly in regions that contained collapsed airspaces.

Despite routine use of antibiotics, some of these chronically ventilated preterm lambs had episodes of generalized bacterial infection, during which their lung lymph flow and L/P increased, indicative of increased pulmonary vascular protein permeability. When their infection resolved in response to appropriate changes in antibiotic therapy, however, lung lymph flow returned to baseline values and L/P decreased to low levels, consistent with increased lung vascular filtration pressure. Thus, the pulmonary edema that typically occurs in chronic lung injury after premature birth and prolonged mechanical ventilation appears to be the result of increased lung vascular filtration pressure rather than increased vascular permeability to protein.

What factors contribute to the abnormal lung fluid balance observed in these chronically ventilated preterm lambs? PVR

was relatively high and did not decrease significantly during the 3 wk of postnatal ventilation. Morphometric studies showed an increase in smooth muscle thickness and elastin around small pulmonary arteries, and a decreased number of lung microvessels and capillary surface density when compared with lambs born at term. These abnormalities reflect a failure of normal vascular modeling during late fetal or early postnatal development, as chronically ventilated lambs had approximately the same amount of smooth muscle in their pulmonary circulation as fetal lambs had at ~125 d gestation, whereas the number of lung microvessels and capillary surface density were significantly reduced when compared with lungs of control lambs that were killed either 1 d or 3 wk after birth at term. It is possible that the increase in lung vascular smooth muscle and microvascular filtration pressure relate, at least in part, to the diminished abundance of endothelial nitric oxide synthase and soluble guanylate cyclase noted in the pulmonary circulation of these lambs (39). It is also likely that the reduced number of lung microvessels through which blood must flow contributes to excessive filtration pressure and accumulation of lung fluid. These abnormalities of lung vascular development—overgrowth of vascular smooth muscle and decreased number of small blood vessels—also have been described in infants with severe BPD (30, 54). It is therefore not surprising that therapeutic strategies designed to reduce fluid filtration pressure in the pulmonary circulation, including fluid and salt restriction (60) and judicious diuretic therapy (61, 62), may help to improve respiratory gas exchange and lung function in babies with CLD.

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## REFERENCES

1. Northway Jr WH, Rosan RC, Porter DY 1967 Pulmonary disease following respiratory therapy of hyaline membrane disease: bronchopulmonary dysplasia. *N Engl J Med* 276:357–368
2. Hislop A, Haworth S 1990 Pulmonary vascular damage in the development of cor pulmonale following hyaline membrane disease. *Pediatr Pulmonol* 9:152–161
3. Abman SH, Wolfe RR, Accurso FJ, Koops BL, Bowman CM, Wiggins JW 1985 Pulmonary vascular response to oxygen in infants with severe BPD. *Pediatrics* 75:80–84
4. Berman W, Yabek SM, Dillon T, Burstein R, Corlew S 1982 Evaluation of infants with BPD using cardiac catheterization. *Pediatrics* 70:708–712
5. Bland RD, Carlton DP 1999 Pulmonary edema in chronic lung disease of early infancy. In: Bland RD, Coalson JJ (eds) *Chronic Lung Disease of Early Infancy*. Marcel Dekker, New York, pp 711–747
6. Carlton DP, Cummings JJ, Scheerer RG, Poulain FR, Bland RD 1990 Lung overexpansion increases pulmonary microvascular protein permeability in young lambs. *J Appl Physiol* 69:577–583
7. Bland RD, Carlton DP, Scheerer RG, Cummings JJ, Chapman DL 1989 Lung fluid balance in lambs before and after premature birth. *J Clin Invest* 84:568–576
8. Bland RD, McMillan DD 1977 Lung fluid dynamics in awake newborn lambs. *J Clin Invest* 60:1107–1115

9. Henry RJ, Sobel C, Berkman S 1957 Interferences of biuret methods for serum protein; use of Benedict's qualitative glucose reagent as a biuret reagent. *Anal Chem* 29:1491-1495
10. Bhutani VK, Sivieri EM, Abbasi S, Shaffer TH 1988 Evaluation of neonatal pulmonary mechanics and energetics: a two factor least mean square analysis. *Pediatr Pulmonol* 4:150-158
11. Albertine KH, Kim BI, Kullama LK, Starcher BC, Cho SC, Carlton DP, Bland RD 1999 Chronic lung injury in preterm lambs. Disordered respiratory tract development. *Am J Respir Crit Care Med* 159:945-958
12. Pinkerton KE, Lewis JF, Rider ED, Peake J, Chen W, Madl AK, Luu RH, Ikegami M, Jobe AH 1994 Lung parenchyma and type II cell morphometrics: effect of surfactant treatment on preterm ventilated lamb lungs. *J Appl Physiol* 77:1953-1960
13. Bolender RP, Hyde DM, Dehoff RT 1993 Quantitative morphology of the lung: a new generation of tools and experiments for organ, tissue, cell and molecular biology. *Am J Physiol* 265:L521-L548
14. Carlton DP, Albertine KH, Cho SC, Davis PL, Long M, Bland RD 1997 Role of neutrophils in lung vascular injury and edema after premature birth in lambs. *J Appl Physiol* 83:1307-1317
15. Shoukri MM, Paus CA 1999 *Statistical Methods for Health Sciences*, 2nd Ed. CRC Press, New York, pp 277-323
16. Zar JH 1984 *Biostatistical Analysis*, 2nd Ed. Prentice-Hall, Englewood Cliffs, NJ, pp 162-235
17. Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD 1997 Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. *Am J Physiol* 272:L452-L460
18. Pierce R, Albertine K, Starcher B, Bohnsack J, Kullama L, Carlton DP, Bland RD 1997 Lung tropoelastin expression increases in lambs after premature birth and 3 days of mechanical ventilation. *FASEB J* 11:A557(abstr)
19. Escobedo MB, Hilliard JL, Smith F, Meredith K, Walsh W, Johnson D, Coalson JJ, Kuehl TJ, Null DM, Robotham JL 1982 A baboon model of bronchopulmonary dysplasia. I. Clinical features. *Exp Mol Pathol* 37:323-334
20. Coalson JJ, Kuehl TJ, Escobedo MB, Hilliard JL, Smith F, Meredith K, Null DM, Jr, Walsh W, Johnson D, Robotham JL 1982 A baboon model of bronchopulmonary dysplasia. II. Pathologic features. *Exp Mol Pathol* 37:335-350
21. Coalson JJ, Kuehl TJ, Prihoda TJ, deLemos RA 1988 Diffuse alveolar damage in the evolution of bronchopulmonary dysplasia in the baboon. *Pediatr Res* 24:357-366
22. Coalson JJ, Winter VT, Gerstmann DR, Idell S, King RJ, deLemos RA 1992 Pathophysiologic, morphometric, and biochemical studies of the preterm baboon with bronchopulmonary dysplasia. *Am Rev Respir Dis* 145:872-881
23. Coalson JJ, Winter V, deLemos RA 1995 Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 152:640-646
24. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA 1999 Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med* 160:1333-1346
25. Bonikos DS, Bensch KG, Northway Jr WH, Edwards DK 1976 Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. *Hum Pathol* 7:643-666
26. Chambers HM, van Velzen D 1989 Ventilator-related pathology in the extremely immature lung. *Pathology* 21:79-83
27. Van Lierde S, Cornelis A, Devlieger H, Moerman P 1991 Different patterns of pulmonary sequelae after hyaline membrane disease: heterogeneity of bronchopulmonary dysplasia? *Biol Neonate* 60:152-162
28. Margraf LR, Tomashefski JF, Bruce MC, Dahms BB 1991 Morphometric analysis of the lung in bronchopulmonary dysplasia. *Am Rev Respir Dis* 143:391-400
29. Bruce MC, Schuyler M, Martin RJ, Starcher BC, Tomashefski JF, Wedig KE 1992 Risk factors for the degradation of lung elastic fibers in the ventilated neonate. *Am Rev Respir Dis* 146:204-212
30. Hislop AA, Wigglesworth JS, Desai R, Aber V 1987 The effects of preterm delivery and mechanical ventilation on human lung growth. *Early Hum Dev* 15:147-164
31. Morray JP, Fox WW, Ketrick RG, Downes JJ 1982 Improvement in lung mechanics as a function of age in the infant with severe bronchopulmonary dysplasia. *Pediatr Res* 16:290-294
32. Gerhardt T, Hehre D, Feller R, Reifenberg L, Bancalari E 1987 Serial determination of pulmonary function in infants with chronic lung disease. *J Pediatr* 110:448-456
33. Rojas MA, Gonzalez A, Bancalari E, Claire N, Poole C, Silva-Neto G 1995 Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. *J Pediatr* 126:605-610
34. Allen K, Haworth S 1986 Impaired adaptation of pulmonary circulation to extrauterine life in newborn pigs exposed to hypoxia: an ultrastructural study. *J Pathol* 150:205-212
35. Jones R, Zapol WM, Reid L 1984 Pulmonary artery remodeling and pulmonary hypertension after exposure to hyperoxia for seven days. A morphometric and hemodynamic study. *Am J Pathol* 117:273-285
36. Thomae KR, Nakayama DK, Billiar TR, Simmons RL, Pitt BR, Davies P 1995 The effect of nitric oxide on fetal pulmonary artery smooth muscle growth. *J Surg Res* 59:337-343
37. Garg UC, Hassid A 1989 Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83:1774-1777
38. Lei PS, Albertine KH, MacRitchie AN, Carlton DP, Bland RD 1998 Guanylate cyclase expression by pulmonary vascular smooth muscle is decreased in chronic lung injury in preterm lambs. *J Invest Med* 46:121A(abstr)
39. MacRitchie AN, Albertine KH, Qu K, Carlton DP, Bland RD 1998 Regulation of endothelial nitric oxide synthase expression in small pulmonary arteries and airways of chronically ventilated preterm lambs. *Pediatr Res* 43:290A(abstr)
40. Shaul PW, Yuhanna IS, German Z, Chen Z, Steinhorn RN, Morin FC 1997 Pulmonary endothelial NO synthase gene expression is decreased in fetal lambs with pulmonary hypertension. *Am J Physiol* 272:L1005-L1012
41. Bland RD, Kullama LK, Day RW, Carlton DP, MacRitchie AN, Albertine KH 1997 Nitric oxide inhalation decreases pulmonary vascular resistance in preterm lambs with evolving chronic lung. *Pediatr Res* 40:247A(abstr)
42. Bland R, Carlton D, Albertine K, Kullama L, Day R, MacRitchie A, Lagerquist K 1998 Pulmonary vascular effects of nitric oxide and cGMP in preterm lambs with chronic lung injury. *FASEB J* 12:A645(abstr)
43. Merritt TA, Cochrane CG, Holcomb K, Bohl B, Hallman M, Strayer D, Edwards DK, Gluck L 1983 Elastase and  $\alpha$ -proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. *J Clin Invest* 72:656-666
44. Ogdan BE, Murphy SA, Saunders GC, Pathak D, Johnson JD 1984 Neonatal lung neutrophils and elastase/proteinase inhibitor imbalance. *Am Rev Respir Dis* 130:817-821
45. Schellenberg JC, Liggins GC 1987 Elastin and collagen in the fetal sheep lung. I. Ontogenesis. *Pediatr Res* 22:335-338
46. Willet KE, McMenamin P, Pinkerton KE, Ikegami M, Jobe AH, Gurrin L, Sly PD 1999 Lung morphometry and collagen and elastin content: changes during normal development and after prenatal hormone exposure in sheep. *Pediatr Res* 45:615-625
47. Hislop A, Reid L 1973 Pulmonary arterial development during childhood: branching pattern and structure. *Thorax* 28:129-135
48. Bruce MC, Bruce EN, Janiga K, Chetty A 1993 Hyperoxic exposure of developing rat lung decreases tropoelastin mRNA levels that rebound postexposure. *Am J Physiol* 265:L293-L300
49. Carlton DP, Cho S-C, Davis P, Bland RD 1994 Inflation pressure and lung vascular injury in preterm lambs. *Chest* 105:115S-116S
50. Bland RD, Hansen TN, Hazinski TA, Haberkern CM, Bressack MA 1982 Studies of lung fluid balance in newborn lambs. *Ann N Y Acad Sci* 384:126-145
51. Jobe A, Ikegami M, Jacobs H, Jones S, Conaway D 1983 Permeability of premature lamb lungs to protein and the effect of surfactant on that permeability. *J Appl Physiol* 55:169-176
52. Jefferies AL, Coates G, O'Brodovich H 1984 Pulmonary epithelial permeability in hyaline-membrane disease. *N Engl J Med* 311:1075-1080
53. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP 1994 Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluid of high-risk preterm neonates. *Pediatrics* 93:712-718
54. Bush A, Busst CM, Knight WB, Hislop AA, Haworth SG, Shinebourne EA 1990 Changes in pulmonary circulation in severe bronchopulmonary dysplasia. *Arch Dis Child* 65:739-745
55. Brigham KL, Woolverton WC, Blake LH, Staub NC 1974 Increased sheep lung vascular permeability caused by *Pseudomonas* bacteremia. *J Clin Invest* 54:792-804
56. Rojas J, Green RS, Helleqvist CG, Olegard R, Brigham KL, Stahlman MT 1981 Studies on group B  $\beta$ -hemolytic *Streptococcus*. II. Effects on pulmonary hemodynamics and vascular permeability in unanesthetized sheep. *Pediatr Res* 15:899-904
57. Bressack MA, McMillan DD, Bland RD 1979 Pulmonary oxygen toxicity: increased microvascular permeability to protein in unanesthetized lambs. *Lymphology* 12:133-139
58. Hazinski TA, Bland RD, Hansen TN, Sedin EG, Goldberg RB 1986 Effect of hypoproteinemia on lung fluid balance in awake newborn lambs. *J Appl Physiol* 61:1139-1148
59. Raj JU 1987 Alveolar liquid pressure measured by micropuncture in isolated lungs of mature and immature fetal rabbits. *J Clin Invest* 79:1579-1588
60. Costarino AT, Gruskay JA, Corcoran L, Polin RA, Baumgart S 1992 Sodium restriction versus daily maintenance replacement in very low birth weight premature neonates: a randomized, blind therapeutic trial. *J Pediatr* 120:99-106
61. Engelhardt B, Elliott S, Hazinski TA 1986 Short- and long-term effects of furosemide on lung function in infants with bronchopulmonary dysplasia. *J Pediatr* 109:1034-1039
62. Albersheim SG, Solimano AG, Sharma AK, Smyth JA, Rotschild A, Wood BJ, Sheps SB 1989 Randomized, double-blind, controlled trial of long-term diuretic therapy for bronchopulmonary dysplasia. *J Pediatr* 115:615-620