Elastase and alpha 1-proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. Role of inflammation in the pathogenesis of bronchopulmonary dysplasia.

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Research Article

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FIGURE 6

Elastase and α_1-Proteinase Inhibitor Activity in Tracheal Aspirates during Respiratory Distress Syndrome

ROLE OF INFLAMMATION IN THE PATHOGENESIS OF BRONCHOPULMONARY DYSPLASIA

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ABSTRACT Pulmonary effluent samples were obtained from 26 preterm or term infants throughout the period of endotracheal intubation. Infants with respiratory distress syndrome, infants with this disorder developing bronchopulmonary dysplasia, and intubated infants without lung disease were compared daily in terms of lung effluent cellularity, albumin, elastase activity, α_1-proteinase content and activity, and elastase inhibitory capacity. The elastase activity was determined to be neutrophilic in origin. Polyacrylamide gel electrophoresis of pulmonary effluents from two infants with respiratory distress syndrome and exposed to $FiO_2 \geq 0.6$ up to 6 d revealed cleavage of α_1-proteinase inhibitor to a 47,000-mol weight fragment suggestive of oxidation. Pulmonary effluent neutrophils, macrophages, and elastase activity were increased by day 3 of life in infants with respiratory distress syndrome eventually developing bronchopulmonary dysplasia. Elastase inhibitory capacity and α_1-proteinase inhibitor activity were reduced in infants developing chronic lung disease. Bronchopulmonary dysplasia developed in infants with enhanced inflammatory response, but with less or inhibited antiprotease activity.

INTRODUCTION

Resolution of severe neonatal respiratory distress syndrome (RDS)[1] may be manifested by recovery of normal lung function and structure, but may be instead associated with epithelial metaplasia of the bronchioles, interstitial and peribronchial smooth muscle proliferation, alveolar inflammation, and interstitial fibrosis with lobular distention by emphysematous foci (1). Clinically affected infants require prolonged oxygen therapy because of hypoxemia and have a postneonatal course often complicated by hypercapnia, cor pulmonale, and frequent respiratory infections (2). 5–30% of infants with RDS develop radiographic evidence of this chronic lung disease. The disease, termed bronchopulmonary dysplasia (BPD) (3, 4) exhibits well-defined radiographic (5, 6), pathologic (7), and cytologic features (8).

Although histopathologic studies of infants dying from RDS document an evolving pattern of pulmonary inflammation during the first week before demise (9), current advances in neonatal ventilatory care have dramatically decreased the number of very premature infants who die from RDS. Despite these advances, it

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[1] *Abbreviations used in this paper:* BPD, bronchopulmonary dysplasia; DFP, diisopropylfluorophosphate; α_1PI, α_1-proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; RDS, respiratory distress syndrome; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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is not possible to predict the outcome of ventilatory therapy in terms of the development of BPD because precise measures are unavailable that predict the extent of lung injury from prolonged oxygen therapy, ventilation with high mean airway pressures, pulmonary plethora from patent ductus arteriosus, or pulmonary airleaks.

Attempts to lessen the frequency and severity of BPD have been unsuccessful, possibly, in part, because of imperfect understanding of the pathogenetic factors that produce lung injury. Although the antioxidant vitamin E has a theoretical basis in reducing lipid peroxidation (10) that justified clinical trials, early enthusiasm for this agent (11) has been unsupported in further studies (12, 13).

Evidence that inflammatory cells are abundant in the lung effluent of infants ventilated for RDS (14, 15) has suggested a role of such cells in lung injury. Animal studies using hyperoxic environments have suggested that the resulting edematous lung injury can be lessened or prevented with neutrophil depletion using a variety of agents (16, 17). Studies on lung injury models related to adult RDS suggest that toxic oxygen radicals may be derived from neutrophils stimulated to release superoxide anion, arachidonate metabolites produced by the activated neutrophils, and from exposure to the reduction of molecular oxygen used in the treatment of the disorder.

Recent investigations of bronchoalveolar lavage in adults with RDS have demonstrated high concentrations of neutrophilic elastase in most patients as well as a reduction in the concentration of "active" α_1-protease inhibitor (α_1PI) in lavage material (19, 20). The inactive α_1PI was found to have been oxidized (21). Observations by Bruce et al. (22) suggest that infants exposed to high FiO_2 concentrations (>0.4) for 3–4 d required greater amounts of α_1PI to porcine pancreatic elastase (a measure of elastase inhibition) to achieve a 15% inhibition of elastase than infants exposed up to 9 d to lower FiO_2 ($\leq$0.4). Furthermore, Bruce et al., using dithiothreitol reduction, demonstrated reactivation of oxidized α_1PI obtained from airway secretions of 12 of 14 infants (23).

Ogden et al. (15) examined serial bronchoalveolar "lavages" in 23 infants during the course of RDS and found that neutrophilic influx in infants with RDS peaked at 4 d and was higher (210×10^6/ml vs. 9.9×10^6) than in ill infants developing BPD. Infants who developed BPD, however, had persistently elevated neutrophil counts for up to 6 wk. Elastase activity in pulmonary lavage fluid peaked at 4 d in neonates with RDS, a finding similar to the data of Merritt (24), though infants developing BPD had elastase levels that peaked at 2 wk of age, in contrast to other reports of similar elastase activity persisting for up to 36 d of life.

The purpose of this study was to investigate lung effluent inflammatory cells, their release of protease, and their influence on the antiprotease system in infants with RDS who recovered normally and in infants who developed BPD.

METHODS

This study was approved by the Committee for Research Involving Humans at the University of California, San Diego, School of Medicine. Gestational age assessment was performed using the technique of Dubowitz and Dubowitz (25).

Severe RDS was characterized by the requirement for supplemental oxygen, retractions of the chest wall, and expiratory grunting (26). The chest radiograph demonstrated a typical reticulogranular pattern with air bronchograms or pulmonary "white-out" obscuring the heart borders (27). Within the first hours of life, the infants with severe RDS required $FiO_2 > 0.6$ because of arterial oxygen pressure (PaO_2) < 50 and/or a arterial CO_2 pressure ($PaCO_2$) > 60 torr, thus requiring endotracheal intubation and mechanical ventilation. BPD was identified using both tracheal aspirate cytologic features and radiographic evidence of the disorder (6, 8).

Infants with conditions requiring endotracheal intubation and assisted ventilation, but generally free of lung disease, constituted a comparison group. These included patients with gastroschisis, birth trauma with hemidiaphragmatic paralysis, asphyxia, ascites from congenital renal disease, and preterm infants requiring mechanical ventilation for apnea. In all infants with RDS, amniotic fluid or tracheal effluent phospholipid profiles documenting lecithin/sphingomyelin ratios < 2.0 and absence of phosphatidylglycerol were obtained (28). Infants included in this report had tracheal effluent and blood cultures obtained soon after admission. No infant included in this report had initial positive blood or tracheal aspirate cultures. All infants with an initial diagnosis of RDS, however, received parenteral ampicillin and gentamicin. To reduce the potential influence of adult α_1PI entering the airspaces by transudation of plasma, infants receiving exchange transfusions were excluded from this study; however, many infants received one or more transfusions of packed red blood cells for ongoing clinical care. These transfusions did not exceed 10% of the calculated blood volume in any 24-h period.

Infants studied had orotracheal intubation with endotracheal tubes (Portex, Inc., Wilmington, MA) of i.d. 3.0–3.5 mm; position of the distal tube end was confirmed radiographically. When clinically indicated for pulmonary toilet, tracheobronchial aspirations were performed by instillation of 0.5 to 1.5 ml of 0.9% NaCl. Suction catheters (No. 6F and 8F) were inserted 1–2 cm beyond the distal endotracheal tube tip and lung secretions suctioned. Aspirates performed solely for clinical indications were collected in Leuken's traps (Cheesbourgh Ponds, Greenwich CT). To rinse the aspirates from the side wall of the catheter, 3 ml of saline were aspirated through the catheter into the trap. Each aspirate was refrigerated at 4°C before processing, usually within 4 h of collection. From one to four aspirates obtained over an 8–12-h period were combined, thus composing each daily sample. Aspirates were centrifuged at 1,000 *g* for 10 min at

4°C. The supernatant was frozen at −70°C until analyzed. The cell pellet was resuspended in isotonic saline and cell counts obtained using hemocytometer after staining with 2% trypan blue. An aliquot of resuspended cells were fixed in ethanol/Hanks' balanced salt solution (1:1) for cytologic fixation and staining according to the Papanicolaou method. Cellular smears were also made by placing cells on albuminized slides and staining with Diff-Quik (Dade Diagnostics, Aquala, PR) for differential counts.

Chemicals, reagents, and antibodies. Agarose M and barbital buffer, pH 8.6, were obtained from LKB (Stockholm, Sweden). Goat anti-human α_1-antitrypsin and rabbit anti-human albumin antibodies were obtained from Cappel Laboratories (Cochraneville, PA). Rabbit anti-human neutrophil elastase antibodies were a generous gift from Dr. Charles Cochrane (La Jolla, CA). Human albumin, Tris buffer, 1, 10 phenenthroline-ethylenediamine tetraacetic acid, porcine pancreatic elastase, type III, *N*-methyl-2-pyrrolidinone, trypan blue, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Succinyl-L-(alanyl)$_3$-*p*-nitroanilide, phenylmethylsulfonyl fluoride (PMSF), and diisopropylfluorophosphate (DFP) were obtained from Calbiochem-Behring Corp., American Hoechst Corp. (La Jolla, CA). Methoxysuccinyl-L-(alanyl)$_2$-prolyl-L-valyl-*p*-nitroanilide was obtained from Vega-Fox Biochemicals, Division of Newberry Energy Corp. (Tucson, AZ). Coomssie Brilliant Blue R-250 was purchased from Eastman Kodak Co. (Rochester, NY), and 0.9% sodium chloride was obtained from Travenol Laboratories (Deerfield, IL).

Concentrations and analysis of individual proteins. The concentrations of pulmonary effluent albumin and α_1PI were obtained by quantitative rocket immunoelectrophoresis according to the technique of Laurell and Erickson (29). The respective antibodies were incorporated at appropriate dilution in 1% agarose and poured onto leveled glass plates. Wells, cut by template, were filled with 5 μl pulmonary effluent or with varied concentrations of purified albumin or α_1PI. Immunoelectrophoresis occurred using 20 V/cm at 24 mA (LKB) for 3 h at 4°C using a circulating antifreeze bath beneath the agarose gels. Following electrophoresis, the agarose gels were eluted with saline for 20 min and then dried in a standard manner, stained using 0.1% Coomassie Brilliant Blue R-250, and destained in 10% glacial acetic acid in ethanol. Following partial drying, each gel was placed on Gel-Bond (Bioproducts, Inc., Warrenton, OR) for preservation.

α_1PI was purified from infant pulmonary effluent according to the technique of Cochrane et al. (21). Briefly, immunopurified goat anti-α_1PI antibody was covalently coupled to cyanogen bromide-activated Sepharose 4B beads. Pulmonary effluent was diluted to contain 92 μg/ml of α_1PI, a quantity necessary for detection by staining, in a slurry of Sepharose 4B containing covalently coupled anti-α_1PI and incubated at 25°C for 1 h. The beads were centrifuged and washed four times with 1 M NaCl in phosphate-buffered saline. The washed beads were eluted with 15 μl 10% SDS for 3 min at 100°C. After centrifugation of the beads, supernatant samples containing native elastase, bound and cleaved α_1PI, were analyzed by electrophoresis in polyacrylamide gels containing SDS according to the method of Laemmli (30). Gels were fixed and stained using 0.1% Coomassie Brilliant Blue R-250.

Elastase activity. Elastase activity was determined in pulmonary effluent by the elastin-agar plate method (31) and by using cleavage of methylosysuccinyl-L-(alanyl)$_3$-prolyl-valyl-*p*-nitroanilide peptide substrate according to the technique of Yusutake and Powers (32). Briefly, 20 μl of pulmonary effluent was reacted with 0.3 mM peptide substrate in 0.2 M Tris with 1 mg/ml of bovine serum albumin (pH 8.0). After 15 min incubation, the reaction was stopped using 500 μl of 1 N acetic acid, and the change in optical density at 410 nm vs. a blank was measured using Beckman DU2 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) with a Gilford 6051 recorder (Gilford Instrument Laboratories, Inc., Oberlin, OH).

Serine elastase activity was determined by measurement of the change in optical density at 410 nm between the initial elastase activity and after preincubation of pulmonary effluent with 10 mM DFP or PMSF for 30 min at 37°C. Inhibition of metalloelastase activity in pulmonary effluent using 10 mM EDTA and 10 mM, 1,10 phenathroline was similarly measured.[2]

Elastase inhibitory capacity. The capacity of pulmonary effluent to inhibit elastase activity was measured in tracheal aspirates of infants daily. All assays were run in duplicate. Briefly, lung effluent (100 μl) was added to 0.2 M Tris buffer with 0.1 M $CaCl_2$ (pH 8.0). The inhibition of porcine pancreatic elastase activity against the peptide substrate 26.6 mM *N*-succinyl-L-(alanyl)$_3$-*p*-nitroanilide in 0.92% *N*-methyl-2-pyrrolidinone was determined after a 2-h incubation at 37°C. The reaction was halted by addition of 200 μl 1 N acetic acid and the differences in activities determined spectrophotometrically at 410 nm. Elastase inhibitory capacity was calculated as the percent reduction of 1.0 μg/ml porcine pancreatic elastase plus endogenous elastase activity determined in pulmonary effluent (30).

Statistical analysis of the pulmonary effluent studies with the unpaired Student's *t* test was used to compare data among the groups. A significant difference was assumed for a $P < 0.05$ on the unpaired test. Linear regression analysis using the statistical module of a Texas Instruments 59 calculator (Texas Instruments, Inc., Dallas, TX) was used to compare cell number and elastase activity.

RESULTS

Patient population. Table I lists characteristics of infants included in this report. Eight infants without primary lung disease were considered as "control" infants becuse they did not have RDS, chronic lung disease, or pneumonia, yet required endotracheal intubation and mechanical ventilation. Two of these infants were born at term and a single infant was exposed to general anesthesia (Halothane) for surgical repair of gastroschisis. No infant in this group had a pulmonary airleak.

12 infants had RDS and had an uneventful recovery of pulmonary function without radiographic evidence of BPD. Three of these RDS infants had pulmonary airleaks. Six additional infants with RDS, all having one or more airleaks, developed BPD. There was no significant difference between these two groups in terms of birth weight, gestational age, or Apgar scores.

[2] Powers, J. Personal communication.

TABLE I
Patient Population Data

BW		GA	Apgar scores at 1, 5 min	Airleak	Primary condition	FiO_2			IMW
g		*wk*				*≥0.8*	*≥0.6*	*≥0.4*	*Total h*
Control infants									
1,830		35	1, 5	0	Asphyxial birth injury	0	0	0	90.8
4,300		41	9, 9	0	Gastroschisis	0.8	29.0	72.0	102.8
2,060		36	4, 7	0	Ascites	2.0	14.0	28.0	52
1,370		31	6, 8	0	Apnea	1.5	50.3	32.1	139.6
1,693		33	7, 8	0	Apnea	0	0	0	43
700		27	4, 6	0	Apnea	2.5	5.4	6.2	255.9
1,560		34	5, 7	0	Birth injury	0	0	39	52
2,670		37	8, 9	0	Seizures, apnea	0	2	29	62
Mean±1 SD	2,023	34				0.85	12.6	25.8	99.8
	±1,078	±4.2				±1.1	±18.3	±14.1	±70.9
RDS infants									
2,270		34	8, 9	0		2.0	3.0	46.0	164.5
1,480		29	7, 8	Pneumothorax, PIE		13.2	64.8	27.6	159.3
1,110		31	5, 6	0		2.2	49.6	73.6	112.8
1,120		27	6, 5	0		4.2	2.5	49.4	129.7
1,370		31	1, 4	0		9.0	29.5	58.4	122.6
1,500		31	1, 4	0		11.2	19.0	73.5	132.6
1,280		31	2, 6	0		10.8	35.0	15.3	97.8
2,240		33	6, 9	0		66.0	7.8	24.5	147.2
1,900		34	−, 7	0		3.5	23.0	10.5	52.0
640		27	2, 6	PIE		67	15.2	20.2	175.5
1,267		30	8, 9	PIE		34	87.5	51.5	191.5
1,338		29	4, 8	0		0	1.8	61.5	139.2
Mean±1 SD	1,510	30.6				18.6	28.2	42.7	135.4
	±455	±3.3				±24.1	±27.0	±22.3	±37.4
RDS-BPD infants									
890		27	2, 4	Pneumothorax		0.8	27.8	114.5	199.5
1,070		28	1, 6	PIE		2.0	1.2	43.5	183.4
1,680		33	4, 5	Pneumothorax		48.0	40.5	30.2	140.8
1,280		30	6, 7	Pneumothorax, PIE		8.5	8.6	37.8	328.2
1,260		29	1, 3	Pneumothorax, PIE		111.0	60.2	220.5	649.0
1,212		29	1, 4	PIE		40.0	50.5	80.8	641.5
Mean±1 SD	1,232	29.3				35.1	31.5	87.9	357.1
	±264	±2.1				±42.2	±23.3	±72.3	±231.8

Infants composing the three groups are listed. Exposure to FiO_2 ≥0.8, ≥0.6, ≥0.4 are listed in hours and total hours of intermittent mandatory ventilation (IMV) regardless of FiO_2 listed. BW, birth weight; GA, gestational age; PIE, pulmonary interstitial emphysema.

Radiologic diagnosis of BPD occurred at 23±6 d (mean±1 SD) of age. The total duration of intermittent mandatory ventilation was significantly longer in the infants developing BPD compared with RDS infants ($P < 0.025$) as was their exposure to $FiO_2 > 0.4$ ($P < 0.05$). All infants with BPD required >30 d of supplemental oxygen, but all six infants were discharged on room air without echocardiographic evidence of cor pulmonale.

Cytology. Analysis of inflammatory cell populations, primarily neutrophilic leukocytes and alveolar macrophages, from lung effluent in infants with RDS

and RDS-BPD compared with infants without primary lung disease reveal that at matched daily intervals, striking differences existed. By the third day of life, infants eventually developing BPD had a nearly 100-fold increase in tracheal effluent cells over control infants and a 10-fold increase in cell number above the RDS group. In comparison, infants with RDS had significantly increased lung inflammatory cells on day 1, 3, 4, and 5 compared with control infants (Fig. 1). Differential cell counts revealed that neutrophils composed 90% of the inflammatory cells while alveolar macrophages and mononuclear cells combined represented only 10% of the inflammatory cell population during the first 3 d of life. By 7 d of age, RDS infants had 4.8 (±1.2) × 10^5 cells/ml, while infants eventually developing BPD had a consistent 20-fold elevation 9.3 (±2.3) × 10^6 cells/ml. By 2 wk of age, the infants developing BPD had a 10-fold increase in exfoliated inflammatory cells when compared with infants with resolving or resolved RDS. A single control infant without lung disease remained intubated at 10 and 14 d. During this second week of life, lung effluent inflammatory cells ranged from 0.85 to 2.7 × 10^6 cells/ml, most likely representing inflammatory changes secondary to intubation. Although daily bacterial and viral cultures of pulmonary effluent in the three groups was not performed, no infant clinically developed pneumonia.

Elastase activity. Pulmonary effluent from intubated infants was analyzed daily for elastase activity. From one to four sequential aspirates were pooled for each measurement and daily measurements were performed in duplicate. Because aspirate volume varied, it was necessary for longitudinal and group comparisons to be expressed as elastase activity per milligram albumin. Albumin concentrations varied only slightly between groups (Fig. 2). Pulmonary albumin concentrations were significantly higher on day 1, possibly because of the inclusion of term infants in this sample. Albumin concentrations in lung effluent were not statistically different in the first week between RDS infants and those developing BPD.

Comparison of elastase activity among control infants during the first week of life revealed rather constant and low levels (>5.0 μg/mg albumin). In comparison, infants with RDS demonstrated a twofold rise in elastase concentrations during the first 5 d of life, returning to control levels by the end of the first week. Infants destined to develop BPD had consistently elevated elastase levels (up to 20-fold) over control infants and fourfold over RDS infants during the first

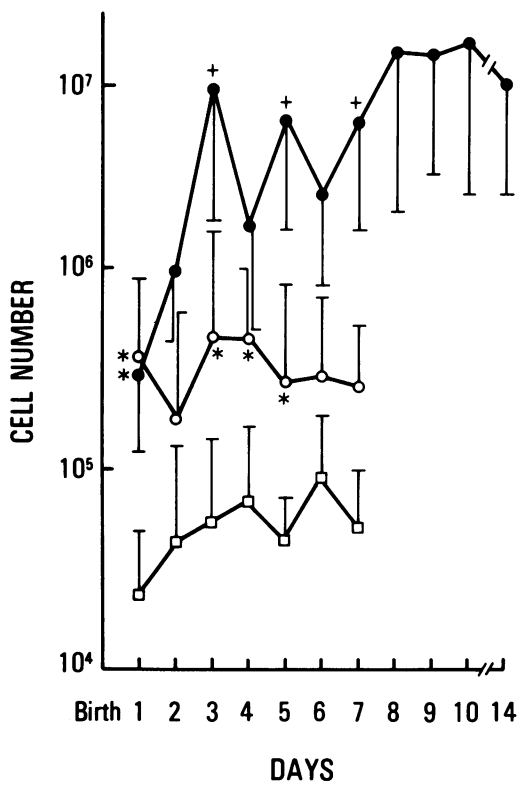

FIGURE 1 Pulmonary effluent inflammatory cells per milliliter effluent on each postnatal day. Cell numbers in RDS (○, n = 12) and RDS-BPD (●, n = 6) groups are compared with control (□, n = 8) infants. Data represent mean±SEM. $^*P < 0.05$; $^+P < 0.01$.

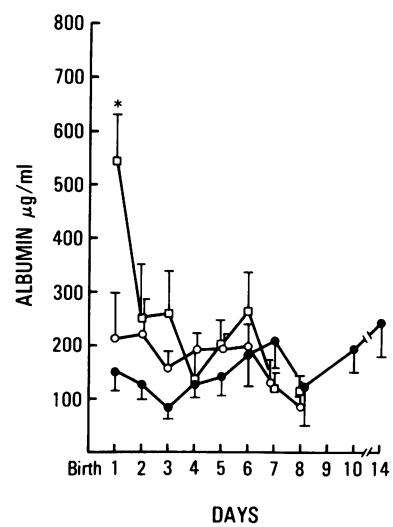

FIGURE 2 A comparison of pulmonary effluent albumin concentration among groups. Data represent mean±SEM. Control infants: □, n = 8; RDS infants: ○, n = 12; RDS-BPD infants: ●, n = 6.
$^*P < 0.05$.

days of life (Fig. 3). Elevations in elastase activity in BPD infants often persisted for up to 2–3 wk of life, reaching levels of 63 μg/mg albumin. In each infant significant elevations in elastase activity occurred on the third day of life (>0.5–33.7 elastase μg/mg albumin) at a time that antedated any clinical, cytologic, or radiographic evidence of BPD.

Elastolysis of elastin particles (Alphasin plates, No. A622, Elastin Products, Pacific, MO) by infant pulmonary effluent could be demonstrated by incubation at 10 μl of effluent in precut wells after 24–48 h at 37°C in a humidified chamber. Although documenting elastolysis of nuchal elastin, this method proved to be only semiquantitative.

To further characterize the cellular origins of the elastase(s) present in the infant pulmonary effluent, double-diffusion immunoprecipitation assays with rabbit-derived antibody to human neutrophilic elastase, as previously described by McGuire et al. (19), revealed a distinct precipitin band from effluent containing 15 μg elastase/mg albumin. Subsequent analysis of 10 samples from infants with RDS demonstrated that preincubation with either 10 mM DFP or PMSF resulted in a variable inhibition (75±29%) of elastase activity. Inhibition of peptide substrate determined elastase activity by 10 mM, 1,10 phenathroline was 20±9%, while 10 mM EDTA inhibited elastase activities >10%. These data suggest that serine elastase, presumably derived from neutrophilic leukocytes, was found in greater proportion than metalloelastase. Recent evidence (33), however, suggests that serine elastase may also be produced by monocytes within the lung and this cell cannot be excluded as contributing serine protease to the activity in pulmonary effluent.

Because we observed a simultaneous increase in pulmonary effluent inflammatory cells and elastase activity, a comparison of inflammatory cell number (cells per cubic millimeter) to elastase activity (micrograms per milligram albumin) was made using all samples derived from all three groups. Analysis of this relationship, using regression analysis, failed to achieve a strong correlation ($r = 0.41$), suggesting that cell number in pulmonary effluent alone was insufficient to predict proteolytic activity.

Elastase inhibitory capacity. The capacity of lung effluent antiprotease to inhibit the activity of porcine pancreatic elastase was compared on daily basis among the infant groups (Fig. 4). A significant reduction in the elastase inhibitory capacity was noted on days 4, 5, and 8 in infants eventually developing chronic lung disease compared with RDS infants. The reduction in elastase inhibitory capacity was still evident at 2 wk of age.

α_1PI levels and activity. Concentrations of α_1PI in pulmonary effluent were determined from the secre-

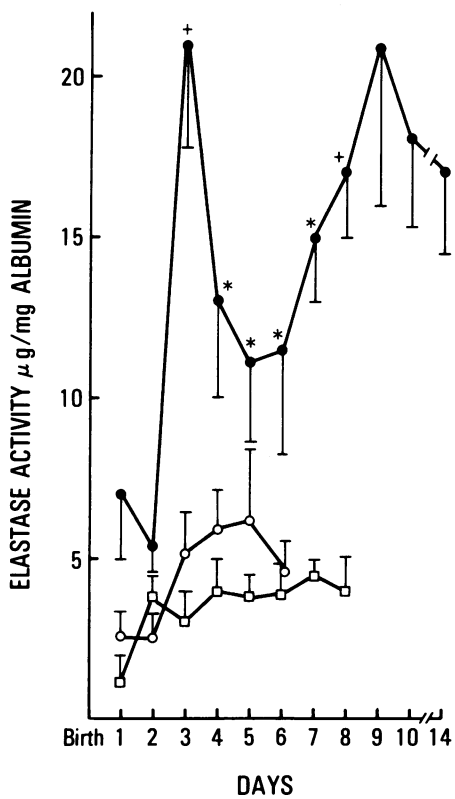

FIGURE 3 Elastase activity measured on each postnatal day is compared among the groups. Data represent mean±SEM. Control infants: □, $n = 8$; RDS infants: ○, $n = 12$; RDS-BPD infants: ●, $n = 6$.
*$P < 0.05$; +$P < 0.01$.

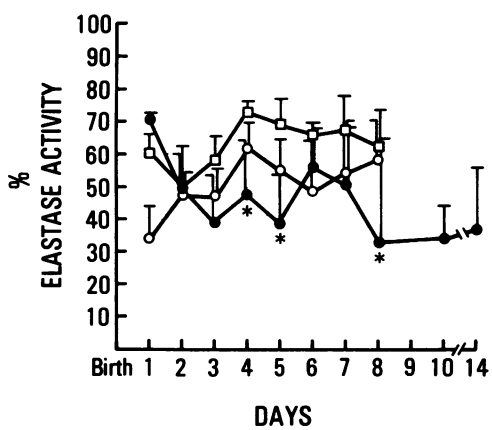

FIGURE 4 Elastase inhibitory capacity of pulmonary effluent among the groups on each postnatal day. Control + 1 SEM, □; RDS + 1 SEM, ○; RDS-BPD + 1 SEM, ●.
*$P < 0.05$.

tions collected daily and compared among the groups. The lower limit of detection was 30 μg/ml pulmonary effluent using the rocket immunoelectrophoresis technique. To account for variations in the concentration of this protein in lung secretions because of variable collections, in terms of volume and dilution by instilled saline and rinsing procedures, we chose to express these levels per milligram albumin (Fig. 5) with 47.8 μg/ml as the lowest detectable concentration. Lung effluent α_1PI per milligram albumin among control infants increased during the first week of life, while in the RDS group this ratio remained somewhat constant (Fig. 5). Infants developing BPD had significantly higher levels on days 2, 3, 8, 10, and 14 of life. The concomitant increase in absolute albumin concentration per milliliter of lung effluent in BPD during the first week of life suggests that an even higher absolute rise in α_1PI level occurred, possibly due to progressive lung inflammation and simultaneous with the significant influx of neutrophils and macrophages into the lung secretions.

The ability of pulmonary effluent α_1PI to alter the activity of porcine pancreatic elastase using succinyl-L-(alanyl)$_3$-*p*-nitroanilide peptide substrate was used to determine the activity of the protein presumably in its nonoxidized state. Table II compares the activity of α_1PI expressed as a percentage of total α_1PI between the RDS infants and the group developing BPD. Using this technique, there was a substantial decline in activity during the first week from 49.1% on day 1 to 20.9% among RDS infants. This decline in $\alpha_1$proteinase activity in BPD infants was consistently lower. The magnitude of these differences were significantly lower on days 2 to 4 in the BPD infants and at the times when pulmonary effluent elastase initially rose in the group of infants developing BPD.

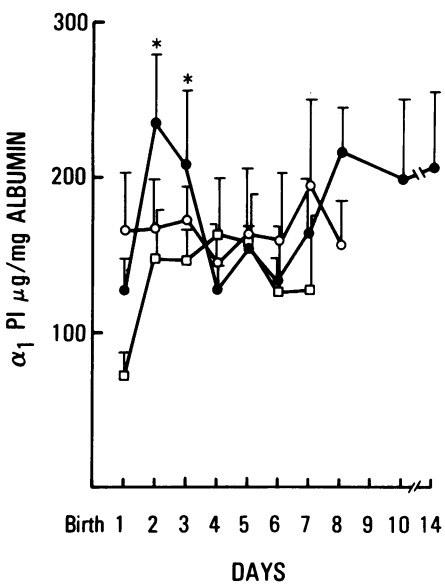

FIGURE 5 Concentration of α_1PI per milligram albumin is compared among the three groups. Control + 1 SEM: □, n = 8; RDS + 1 SEM: ○, n = 12; RDS-BPD + 1 SEM: ●, n = 6.
*$P < 0.05$.

Analysis of inactive α_1PI. Inhibition of proteolytic enzymes including elastase occurs when they bind to α_1PI. Under conditions where the inhibited enzyme is dissociated from the α_1PI, the α_1PI may be demonstrated to have undergone proteolytic cleavage. To evaluate pulmonary effluent for this phenomenon, α_1PI in tracheal aspirate fluids obtained on the first day of life was compared to α_1PI obtained after 4 or 6 d of therapy using SDS-polyacrylamide gel electrophoresis (PAGE) and immunoelectrophoresis. In two infants developing BPD, analysis of α_1PI bands differed from day 1 and 4 or 6 d after oxygen therapy and mechanical ventilation for RDS. On the first day of treatment in both infants, a single protein band at 52,000 D was detected (Fig. 6). After 4 or 6 d of treatment and coincident with <50% active α_1PI, two protein bands were observed, one of 47,000 and another of ~80,000 D. Johnson and Travis (34) previously demonstrated that oxidized α_1PI, but not native α_1PI, underwent cleavage by elastase to yield a 47,000-D fragment. An 80,000-D band also found in the pulmonary effluent has previously been identified in pulmonary effluent of individuals with adult RDS to represent α_1PI-elastase complex (29).

Our finding of inactivated α_1PI, cleavage of inactivated, presumably oxidized α_1PI, and elastase-α_1PI complex formation document that antiprotease activity can be substantially altered by the presence of elastase in pulmonary effluent and oxidation, presumably by exposure to high oxygen or neutrophil-derived oxidants.

DISCUSSION

Injury to lung connective tissues, primarily elastin and collagen, results when an imbalance between the elaboration of proteinases and the inhibitory capacity of proteins, primarily α_1-globulins, exists in the lung. Recent studies by Gadek et al. (35) have demonstrated that α_1PI is the predominant inhibitor of neutrophil elastase in the regions of gas exchange in the lung of adult humans.

The precise interplay between immaturity, surfactant deficiency, oxygen exposure, mechanical ventilation, patency of the ductus arteriosus, and other factors in the pathogenesis of BPD has remained elusive. The potential role of lung inflammation accompanied

TABLE II
Percent Activity of α_1PI

	Age (Days)						
	1	2	3	4	5	6	7
RDS (n = 12)	49.1 ±22.8	35.3 ±16.4	39.6 ±11.6	29.1 ±8.8	35.9 ±25.3	31.4 ±5.0	—
RDS-BPD (n = 6)	33.1 ±9.8	13.1* ±14.6	20.3* ±13.7	17.8* ±11.1	31.3 ±19.5	27.4 ±25.8	26.7 ±5.1

α_1 Proteinase inhibitory activity is presented as a percentage of the total α_1PI after reaction with porcine pancreatic elastase.
* $P < 0.05$.

by an influx of polymorphonuclear leukocytes has recently been confirmed by cytologic examination of tracheal effluents by Merritt et al. (8) and Doshi et al. (36). Merritt (24) measured elastase in the lung effluent from a small group of infants with RDS who developed BPD and found that elastase activities in these infants to be higher and sustained when compared with infants in who RDS resolved. This report expands these previous observations to include the interaction between neutrophil-derived elastase, lung effluent proteinase inhibitor, and the mechanisms of the proteinse-antiproteinase imbalance in human preterm infants with RDS developing BPD.

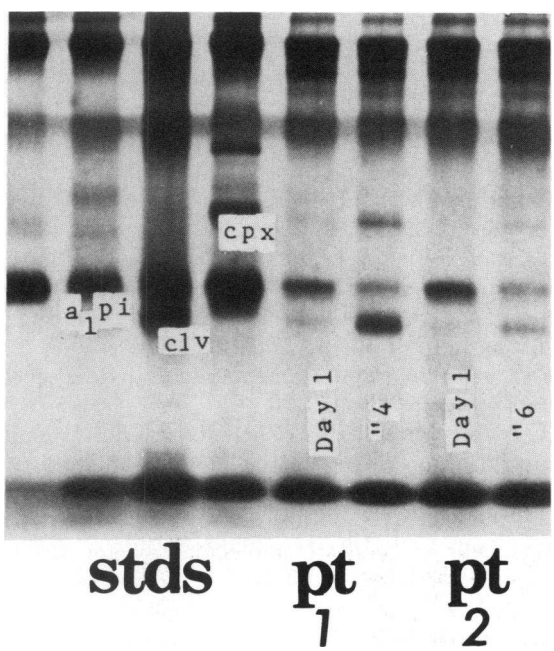

FIGURE 6 SDS-PAGE of pulmonary effluents in two infants. Standard (*stds*) for α_1 PI (*clv*) at 47,000 D, and elastase-α_1 PI complex (*cpx*) at ≅80,000 D have been prepared. Patient (*pt*) 1 on day 1 demonstrates α_1PI but no complex formation. By day 4, this infant has evidence of elastase-α_1PI complex formation and cleaved α_1PI. This infant required 88.5 h FiO_2 ≥ 0.6 at the time of effluent sampling. Pt 2 demonstrates complexed elastase-α_1PI and cleaved α_1PI. This infant remained in FiO_2 > 0.6 at time of sampling on day 6.

Infants with RDS destined to developed BPD were found to have greater numbers of inflammatory cells in pulmonary effluent than infants with RDS alone. Exposure to high ambient oxygen has previously been demonstrated to increase the proportion of neutrophils in lung lavage, as well as alveolar macrophages, in oxygen exposure >0.9 both in the neonatal guinea pig (24) and rats (37) and in tracheal aspirates sequentially obtained in preterm infants (15).

Up to 6% of the fetal or neonatal lung parenchyma is elastin (38). In this study we provide evidence that pulmonary effluent elastase levels are significantly elevated during the first days of life in neonates with RDS who later develop chronic lung disease characterized by structural alterations in the elastin and collagen fibers in the lung when compared with infants with RDS having a normal recovery. This protease activity is primarily neutrophilic in origin. This finding is similar to the reports of Lee et al. (20) and McGuire et al. (19) who identified neutrophil-derived elastase in bronchoalveolar lavage of adults with RDS. Our findings and those of Ogden et al. (15) suggest that similar mechanisms may result in lung injury during infancy. Either surfactant deficiency or treatment modalities for the disorder (high oxygen exposure, intermittent mandatory ventilation, or both) may result in pulmonary inflammation thus elaborating elastase capable of degrading lung elastin when inhibition by α_1PI is insufficient. Infants developing chronic lung disease have a more pronounced inflammatory response or a more reduced capacity to limit this re-

sponse than infants not developing this disorder. Based upon immunologic identification, reaction with a highly specific substrate and elastolysis of native elastin, this study documents that neutrophilic elastase activity measured in lung effluent when unfettered is capable of inducing connective tissue destruction.

Alveolar macrophages were observed to increase from ~2 to 45% of inflammatory cells in tracheal aspirates during the first 2 wk of life in intubated preterm infants being treated for RDS; however, we did not identify substantial metalloelastase activity in pulmonary effluent. Hinman et al. (39) have reported that alveolar macrophages from smokers obtained by bronchoalveolar lavage release calcium-dependent metalloelastase. Further investigation into whether proteinase activities from other inflammatory cells change during chronic lung disease are warranted. That inflammatory cell numbers may not reflect elastase activity is not expected since lysosomal proteases may be secreted by neutrophils and macrophages fixed to tissues and not accessible to airway lavage fluids. Nonetheless, this relationship does suggest that elastase content in pulmonary effluent does reflect the magnitude of inflammatory response and provides a "marker" for the potential injury of lung parenchyma in preterm newborns.

Concomitant with elastase release, α_1PI activity was substantially reduced in infants receiving high concentrations and longer durations and higher concentrations of oxygen exposure and mechanical ventilation. Absolute α_1PI per milligram albumin was generally constant during the first days of life in lung effluent; however, both lung lavage and serum levels (data not shown) of α_1PI activity fell in both RDS and RDS-BPD infants.

These observations, therefore, provide evidence that neutrophilic elastase activity unabated by proteinase inhibitor contributes to the development of chronic lung disease. Inactivation of α_1PI has been demonstrated by Cochrane et al. (21) to occur by complex formation of neutrophilic elastase with α_1PI and proteolytic cleavage of oxidized α_1PI in adults with adult RDS. Using identical SDS-PAGE techniques to evaluate α_1PI oxidation in infant tracheal aspirates, we found in infants developing chronic lung disease that elastase-α_1PI complexes were formed and subsequently cleaved. These α_1PI fragments were identified after 5–6 d of treatment. The presence of this 47,000-Dα_1PI fragment is consistent with evidence from RDS lavage fluids that oxidation of α_1PI, presumably at the methionine peptide residue, and subsequent cleavage after elastase binding, occurs as previously demonstrated by Johnson and Travis (40). Although it is not possible to totally exclude that elastase release, α_1PI oxidation, and α_1PI-elastin complex formation, occurred after the pulmonary secretions were removed from the neonates respiratory tract and maintained at 0°–4°C, immediate analysis after aspiration compared with analysis at 2 and 4 h after maintenance in a cold room varied <10%. Furthermore, elastase activity in infants with less severe RDS was very low when compared with infants with severe lung disease, and α_1PI activity was found at significantly higher levels.

Although the infants developing BPD were exposed significantly longer to varying, but higher levels of FiO_2, the precise etiology of the presumed oxidation occurring in infants' lungs remains unclear. Stimulated neutrophils have been shown to release oxidants sufficient to inactivate α_1-proteinase and to suppress the elastase inhibitory capacity of plasma. Neutrophil myeloperoxidase, together with H_2O_2 and halide, has been shown to inactivate α_1PI at the reactive site methionyl peptide according to Matheson et al. (41).

Pulmonary edema during the first week of life has also been suggested as important in the pathogenesis of BPD. Preterm infants with RDS and immature lambs with oxygen exposure may have increased lung capillary permeability contributing to lung edema. (42). Inappropriate fluid administration resulting in pulmonary edema was suggested by Brown et al. (43) to contribute to chronic lung disease; however, when controlling for daily fluid administration, Merritt et al. (44) found that ductus arteriosus patency, with left-to-right shunting, results in pulmonary edema (necessitating protracted assisted ventilation and higher FiO_2 exposure) and was more frequently associated with BPD and death. Lung edema induced by toxic levels of oxygen has also been considered as contributory to the pathogenesis of chronic lung disease and may require the presence of neutrophils, although adult rabbits depleted of neutrophils have been reported to develop pulmonary edema and die in an environment of FiO_2 of 1.0 in a time frame identical to nondepleted rabbits (45).

Our observation that α_1PI is inactivated presumably by oxidation occurring in the lung parenchyma and airways in infants undergoing treatment for RDS, suggests that oxidants are released by either the inflammatory cells or by direct molecular oxygen reaction within the cells. Elastase binding and subsequent inactivation of α_1PI, thus, result in the inability to neutralize neutrophil-mediated elastase permitting elastin degradation. Pulmonary parenchymal elastin degradation by intratracheal instillation of porcine pancreatic elastase results in emphysematous changes with hyperinflation and interstitial fibrosis consistent with some of the pathologic features of BPD (46). Elastin fragments have been shown to be chemotactic (47) for additional inflammatory cells as well as fibroblasts (48). Oxidants are known to injure pulmonary tissue in sev-

eral ways. Stimulated human neutrophils induce increased vascular permeability in isolated perfused rabbit lungs, while neutrophils from a patient with chronic granumomatous disease fail to generate oxidants after stimulation and did not increase vascular permeability (49). In rats, intrapulmonary glucose, glucose oxidase, and lactoperoxidase induced acute pulmonary injury marked by an increased vascular permeability (50). This injury was inhibited in a dose-dependent manner by catalase. Xanthine and xanthine oxidase instilled intratracheally also causes pulmonary edema with partial inhibition by superoxide dismutase, but not catalase. These foregoing data strongly suggest that intrapulmonary generation of oxidants results in lung injury resulting in changes in vascular permeability previously documented in fetal sheep exposed to oxygen (51).

Exposure to high levels of inspired oxygen have also been implicated in the generation of oxidizing species generated in vivo. Infants developing BPD have been documented to have longer durations of $FiO_2 \geq 0.4$ exposure; and duration of mechanical ventilation was longer in infants developing this disorder. Yet, it remains difficult to establish toxic "doses" of either inspired oxygen or airway pressure because these clinical variables are influenced by initial severity of RDS, differences in management, and fail to account for variability in the inflammatory response and protease inhibitory capacity among a host of varying clinical conditions including ductal left-to-right shunting, fluid administration, and sepsis.

Although additional data are required to clarify the quantitative roles of proteinase release, proteinase inhibition, and oxidant-induced injury in humans, this study documents that pulmonary effluent elastase concentration and proteinase inhibitory capacity can be used to identify infants with a strong chance of developing BPD later in their clinical course. Furthermore, these measures of lung injury may prove useful in evaluation of new modes of RDS intervention including high frequency oscillatory ventilation and surfactant supplementation.

Additional studies are also needed to clarify the role of other antiproteinases including α_2-macroglobulin during oxygen exposure in preterm infants.

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