Persistent Elastase/Proteinase Inhibitor Imbalance during Prolonged Ventilation of Infants with Bronchopulmonary Dysplasia: Evidence for the Role of Nosocomial Infections

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ABSTRACT. Acute imbalance between elastase and α -1-proteinase inhibitor (α 1Pi) may contribute to the development of bronchopulmonary dysplasia (BPD). The question of whether such an imbalance persists in BPD infants still requiring mechanical ventilation after 4 wk of life has not been previously addressed. We studied 14 infants still on mechanical ventilation at 4 wk of age: nine had BPD and five did not. Weekly (4 to 9 wk) serum and bronchoalveolar lavage (BAL) specimens were taken. α 1Pi and α -2-macroglobulin were measured in serum and BAL by immunoturbidimetric assay. BAL elastase activity was measured by cleavage of a synthetic substrate and expressed as ng of porcine pancreatic elastase equivalent. Infants with BPD had higher levels of serum α 1Pi and α -2-macroglobulin than those without BPD. In contrast, the corresponding BAL levels were either similar or even decreased (α 1Pi). Moreover, there was a 3-fold increase in elastase-1Pi imbalance expressed as the BAL ng of porcine pancreatic elastase equivalent/ α 1Pi ratio. The role of nosocomial infections was evident in a subgroup of 11 infected BAL aspirates in BPD infants. In such cases we found a 3-fold increase in the BAL ng of porcine pancreatic elastase equivalent/ α 1Pi ratio as compared to 35 noninfected BAL in BPD infants. These data suggest a persistent alveolitis with imbalance between elastase and proteinase inhibitors in prolonged severe BPD. Such an imbalance is, in part, explained by a local destruction and/or inactivation of α 1Pi. Our results also emphasize the increase in proteolysis with nosocomial pneumonia. (*Pediatr Res* 26: 351-355, 1989)

Abbreviations

α 1Pi, α -1-proteinase inhibitor
 α 2M, α -2-macroglobulin
AM, alveolar macrophage
BAL, bronchoalveolar lavage
BPD, bronchopulmonary dysplasia
MV, mechanical ventilation
NPE, ng of porcine pancreatic elastase equivalent
PMN, Polymorphonuclear
PMSF, phenylmethylsulfonyl fluoride
RDS, respiratory distress syndrome
SAPNA, N-succinyl-L(alanyl 3)-P-nitroanilide in N-methyl-pyrrolidine

Premature infants with acute neonatal lung injury, usually RDS, may develop neonatal chronic pulmonary disease, of which the most common form is BPD. The exact pathogenesis of BPD is unresolved, however, many recent reports emphasize the importance of inflammatory events (1, 2). Histologic and cytologic studies of infants with RDS have shown an early and significant alveolitis which consists of inflammatory cells (PMN and AM) (1-4). This alveolitis is prolonged in infants with RDS who develop BPD (3). Activation of these cells within the lung (5, 6) leads to secretion and release of many products that alter function or damage lung cells and connective tissues. The potential role in the pathogenesis of BPD of one of these products, the main proteolytic enzyme elastase, has recently been demonstrated in infants with RDS between the 2nd and the 28th d of postnatal age. Two studies reported an elevation of BAL elastase levels, coupled with low BAL levels of the main proteinase inhibitor α 1Pi. This results in an imbalance between elastase and α 1Pi (1, 2). Moreover, this elastase-proteinase inhibitor imbalance has been associated with the destruction of lung connective tissue and the resultant fibrotic changes characteristic of BPD (7). The question of whether such a BAL imbalance persists in BPD infants still requiring MV after 28 d of life has not been addressed. Furthermore, prolonged BPD is often associated with nosocomial lung infections, and the role of such infections in elastolytic damage has been recently emphasized in both acute and chronic lung disease (8, 9). We designed a prospective study to answer the following questions: 1) Is severe BPD that still requires mechanical ventilation at d 28 associated with elastase-proteinase inhibitor imbalance? 2) Does MV per se result in such an imbalance? 3) Do nosocomial infections play a role in that imbalance?

MATERIALS AND METHODS

Patient groups. Fourteen low birth wt infants with or without BPD, who still required MV at 28 d of age, were enrolled in the study. The BPD group consisted of nine infants who fulfilled all the following criteria: 1) Primary respiratory disorder requiring MV with supplemental oxygen from birth and for at least 3 d (five infants had RDS, two pneumonia, and two severe wet lung disease); 2) chronic respiratory distress at 28 d of age, requiring MV and supplemental oxygen to maintain $P_{aO_2} > 60$ torr and $P_{aCO_2} < 45$ torr; 3) chest x-ray 28 d postnatally consistent with the diagnosis of BPD stage III or IV according to Northway *et al.* (10). Five infants without BPD but with prolonged MV served as a control group. These infants fulfilled all the following criteria: 1) No primary respiratory disorder, but MV from birth for at least 3 d without supplemental oxygen (the five infants enrolled had MV because of their low birth wt); 2) absence of significant

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chronic respiratory distress, as described above, but necessity for MV (e.g. for apneas) at 28 d of age; 3) normal chest x-ray at 28 d of age.

Study design. After informed consent was obtained from the parents, the 14 patients were studied weekly, from the 4th to the 9th wk of life or until extubation. We performed 1) BAL; 2) blood sampling; 3) recording of clinical data; 4) diagnosis of nosocomial pneumonia. To reduce the potential influence of adult blood transfusion on the results, no measurements were performed for a period of 48 h after transfusion.

Bronchoalveolar lavage. Tracheal effluents were obtained using a standardized technique of BAL (1, 2), by the same physiotherapist as follows: 1 mL of sterile 0.9% NaCl was gently instilled into the endotracheal tube. Then the infant was ventilated for a few breaths and deep endotracheal aspiration performed using a no. 6 French catheter. To rinse the side wall of the catheter, 1 mL of sterile 0.9% NaCl was then aspirated. The product of four lavages at 30-min intervals was collected in a sterile mucus extractor. A total of 4 mL of sterile 0.9% NaCl was instilled. Mean amount of tracheal effluent was not different between groups (3.35 ± 0.09 mL for controls and 3.42 ± 0.05 mL for BPD). Each BAL was refrigerated at 4°C before processing and centrifuged at $1500 \times g$ for 15 min at 4°C as soon as possible. The cell free supernatant was frozen at -20°C until analysis.

Blood samples. One mL of blood was drawn by venous puncture and plasma was separated by centrifugation and frozen within 2 h after sampling until analysis.

Clinical data were recorded in order to assess requirement of oxygen (FiO_2) and of MV, expressed as a ventilatory index (mean airway pressure, $\text{FiO}_2/\text{PaO}_2$).

Diagnosis of nosocomial pneumonia was made when all the following criteria were present: 1) A dominant pathogenic microorganism and altered PMN in routine tracheal aspirate cultures; 2) clinical and radiologic signs of pneumonia; 3) laboratory markers of infection (abnormal values for white blood count, C-reactive protein, and fibrinogen).

BAL elastase activity was determined by the cleavage of a synthetic substrate, SAPNA, as described by Bieth *et al.* (11). Briefly, 100 μL of BAL were reacted with 30 μL of 125 mM SAPNA in 900 μL buffer (200 mM Tris-HCl pH 8.3 with 10 mM CaCl_2). After 1 h incubation at 37°C, the reaction was stopped on ice and the change in optical density at 410 nm measured using an LKB 4050 spectrophotometer (Biochrom Ltd, Cambridge, England). Elastase activity was then compared to the cleavage of SAPNA by porcine pancreatic elastase in the same buffer containing 1 mg/mL of BSA. Measurements were done in duplicate. Elastase activity was expressed as NPE divided by BAL albumin. Serine-elastase inhibitable activity was determined by the change in optical density between the initial elastase activity and that after preincubation of 100 μL of BAL with 0.01 mM PMSF, for 1 h at 37°C. Metallo-elastase inhibitable activity was similarly determined after preincubation with 5 mM EDTA.

BAL and serum albumin and proteinase inhibitors. We measured BAL albumin as an indicator of persistent increase in pulmonary epithelial permeability and also to standardize BAL measurements of elastase and proteinase inhibitors. Albumin and two main proteinase inhibitors, $\alpha 1\text{Pi}$ and $\alpha 2\text{M}$, were assayed by immunoturbidimetry in both BAL and serum specimens using a Kone Progress Autoanalyser (Kone Instrument, Helsinki, Finland). Samples of 2, 12, and 24 μL (for albumin, $\alpha 1\text{Pi}$ and $\alpha 2\text{M}$, respectively) of serum diluted 1:50 in PBS pH 7.4 or of undiluted BAL were mixed with 100 μL of a 45 g/L dilution of polyethylene glycol in PBS and a 1:6 dilution of appropriate antiserum. Standard curves were prepared by serial dilutions of the N protein standard. The lowest detectable concentrations were 5 $\mu\text{g}/\text{mL}$ for albumin, 2.5 $\mu\text{g}/\text{mL}$ for $\alpha 1\text{Pi}$ and 1 $\mu\text{g}/\text{mL}$ for $\alpha 2\text{M}$.

BAL NPE/ $2\alpha 1\text{Pi}$ ratio. To express the imbalance between elastase and the most important proteinase inhibitor, $\alpha 1\text{Pi}$, and because 2 mg $\alpha 1\text{Pi}$ is required to neutralize each mg of elastase,

we used their ratio expressed as $\text{NPE}/2 \mu\text{g } \alpha 1\text{Pi}$ as previously suggested by Ogden *et al.* (2).

Chemical reagents. Porcine pancreatic elastase and SAPNA were obtained from Biosys, Compiègne, France; PMSF and BSA from Sigma Chemical Co., St. Louis, MO; EDTA and buffers from Merck, Darmstadt, FRG; antibodies and standard proteins from Orimbio, Paris, France.

Data analysis. The data are given as mean $\pm$ SD when distributed normally, otherwise as median and range. In our five control infants, values at increasing postnatal ages were not statistically different. We therefore combined the 14 corresponding values. Differences between groups were evaluated with analysis of variance if the data were normally distributed, or with nonparametric tests: Mann-Whitney for unpaired values and Wilcoxon signed rank for paired values, if they were not. $p < 0.05$ was taken as significant.

RESULTS

Patient data. The two groups, BPD ($n = 9$) and control ($n = 5$), had similar birth wt and gestational ages. However, clinical evolution, as shown by requirements at d 1, and wk 4 and 8, of oxygen, FiO_2 , and MV, expressed as a VI, were different (Table 1).

Serum and BAL albumin. Serum albumin levels were not significantly different between control and BPD infants at any sampling time. In contrast, weekly BAL albumin levels were increased in BPD infants. The difference was significant ($p < 0.05$) only at wk 4 when the median (range) was 103 mg/L (33–184) in BPD infants, in comparison to the overall median (range) 32 mg/L (1.4–67) in control infants. However, the latter value was not significantly different from the overall median (range) of 47 mg/L (9–462) for the BPD infants.

Serum and BAL proteinase inhibitor. Table 2 shows that weekly serum levels of the two proteinase inhibitors $\alpha 1\text{Pi}$ and $\alpha 2\text{M}$ were increased in BPD infants as compared to controls. Increases were significant at wk 9 for $\alpha 1\text{Pi}$ and wk 5 and 6 for $\alpha 2\text{M}$, and also for the 46 overall $\alpha 1\text{Pi}$ values. In contrast, the weekly BAL proteinase inhibitor/albumin ratios, in BPD infants appear slightly decreased. However, this decrease was significant only for the 46 overall $\alpha 1\text{Pi}$ values (Table 2).

BAL NPE specific activity and BAL NPE/ $2 \alpha 1\text{Pi}$ ratio. Compared to controls, weekly NPE specific activity in BAL was slightly increased in BPD infants (Table 3). This was significant at wk 9, but not over the whole study period. Nonetheless, there was a 3-fold increase in the BAL NPE/ $2 \alpha 1\text{Pi}$ ratio in BPD infants as compared to controls. This was significant at wk 7, 8, 9, and for all the 46 BPD results combined.

In 31 of 46 BAL samples in BPD infants, we were able to characterize the cellular origin(s) of this elastase activity. PMSF-inhibitable activity (or serine-elastase activity) was detected in 29 of 31 BAL samples [52% (0–87) of total activity] and the percentage was greater ($p < 0.05$) than EDTA-inhibitable activity (or metallo-elastase activity) which was detected in 22 of 31 BAL samples [28% (0–100) of total activity].

Results in subgroup of infected BPD infants. Eleven of the 46 samples in BPD infants were taken during a nosocomial infection as defined above. The results of these 11 samples were combined and compared to the 35 combined results of noninfected BPD infants and to the 14 combined results of the control group. None of the five infants enrolled in the control group had nosocomial infections at the time of samplings. There were no differences in serum levels of albumin and proteinase inhibitors between those subgroups. In the infected BPD subgroup, the albumin level in BAL was elevated, as compared to control group (Table 4). BAL $\alpha 1\text{Pi}$, but not BAL $\alpha 2\text{M}$, was significantly decreased as compared to control group. The BAL NPE/albumin ratio was increased in the infected BPD subgroup, as compared to control group. Moreover, there was a 7-fold increase in the NPE/ $2\alpha 1\text{Pi}$ ratio in the infected BPD subgroup as compared to

Table 1. Birth wt (BW), gestational age (GA), and clinical condition of BPD and control infants on d 1, and wk 4 and 8 of age [Mean (SD)]

	Patient (n)	BW (g)	GA (wk)	Clinical Condition					
				FiO ₂			VI*		
				D 1	Wk 4	Wk 8	D 1	Wk 4	Wk 8
BPD	9	1180 (300)	30 (2.1)	0.42 (0.18)	0.35 (0.09)	0.40 (0.08)	0.11 (0.09)	0.06 (0.03)	0.09 two patients (0.03) extubated
Control	5	1100 (179)	29 (1.2)	0.21 (-)	0.21 (-)	0.21 (-)	0.01 (0.004)	0.02 (0.07)	All patients Extubated

* Ventilatory index = (mean airway pressure × FiO₂)/PaO₂.

Table 2. Serum and BAL α 1 Pi, and α 2 M in control and BPD infants at 4 to 9 wk of age*

Time of lavage (wk)		Controls		BPD					
		Overall 4-7	4	5	6	7	8	9	Overall 4-9
(n of samples)		(14)	(9)	(9)	(9)	(8)	(7)	(4)	(46)
α 1 Pi									
Serum g/L	Median	1.9	2.5	2.2	2.4	2.3	2	2.1	2.2†
	Range	1.3-3.1	1.9-3.8	1.9-3.3	1.7-3.9	1.6-3.3	1.8-2.3	2-2.5	1.6-3.9
BAL μ g/mg albumin	Median	90	66	52	58	64	32	45†	61†
	Range	50-357	17-160	20-152	23-187	25-119	16-237	5-82	5-237
α 2 M									
Serum g/L	Median	2.9	3.1	3.7†	3.6†	3	2.8	3.1	3.1
	Range	2.2-4	2.8-5.3	2.5-6.8	2.8-5.3	2.6-6.3	2.4-3	2.6-8	2.4-8
BAL μ g/mg albumin	Median	61	79	85	82	89	56	77	77
	Range	12-357	12-160	5-120	11-113	11-203	16-212	39-107	5-212

* Median values for each week and for the whole study period in the BPD infants are compared to median values for the whole period in control infants.

† *p* versus control values < 0.05.

Table 3. BAL-free elastolytic sp act (NPE/albumin ratio) and BAL elastase/2 α 1 Pi ratio (NPE/2 α 1 Pi) in control and BPD infants at 4 to 9 wk of age*

Time of lavage (wk)		Controls		BPD					
		Overall 4-7	4	5	6	7	8	9	Overall 4-9
(n of samples)		(14)	(9)	(9)	(9)	(8)	(7)	(4)	(46)
NPE/mg albumin	Median	30	42	42	44	47	39	93†	50
	Range	10-110	18-83	7-215	15-600	12-230	11-230	35-200	7-600
NPE/2 α 1 Pi	Median	0.18	0.25	0.37	0.30	0.62‡	0.36†	2†	0.41‡
	Range	0.04-0.6	0.11-2.4	0.16-2.1	0.08-1.6	0.16-1.9	0.04-8	0.2-6.1	0.04-8

* Results are expressed as in Table 2.

p versus control values, † < 0.05, ‡ 0.01.

the control group and over 3-fold increase as compared to the noninfected BPD subgroup. In 10 of the 11 BAL samples of the infected BPD subgroup, we were able to characterize the cellular origin(s) of the elastase activity. Results are listed in Table 5. PMSF-inhibitable activity (serine-elastase activity) was detected in all the BAL specimens and at a higher percentage (*p* < 0.05) than EDTA-inhibitable activity (or metallo-elastase activity) which was detected only in six samples.

DISCUSSION

We report evidence of a persistent imbalance between elastase and proteinase inhibitors in severe BPD which still required MV at 4 wk of age. These results support the view that subacute pulmonary inflammation plays an important role in the development of BPD. According to histological and cytological descriptions (1-4, 12) alveolitis is present in BPD, in both early and late stages. Many recent reports provide data concerning

inflammatory cells in the tracheal aspirate from infants with RDS. They all report that PMN is the predominant cell type in the early stages (1st to 4th d) of RDS and appears before AM in the lung (1-4), but they disagree about the sequence of cytologic events during the early development of BPD (1st to 4th wk). Merritt *et al.* (3) found that AM was the predominant inflammatory cell type after the 1st wk of evolution, whereas Ogden *et al.* (2) found the PMN count elevated until 5 wk of life. Clement *et al.* (6) suggest that even in older infants with BPD (4 mo of age), who no longer require MV, AM is the predominant cell type and appears to be activated, as in the early stage of BPD (5). In neonatal RDS (1), and in adult RDS (13, 14), the predominant elastase activity is inhibitable by PMSF (serine-elastase), and is demonstrably of PMN origin (PMN-elastase). In chronic pulmonary injury (15), the predominant activity may be inhibitable by EDTA (metallo-elastase), which would probably be of AM origin.

Our results confirm that both PMSF- and EDTA-inhibitable

activities are present in BPD, but the PMSF-inhibitable activity (serine-elastase or PMN-elastase) is the predominant one, even in the infected BPD subgroup. Our observations (Table 5, patients 3, 6, and 7) substantiate those of Bruce *et al.* (9), who reported the presence of predominantly PMSF-inhibitable activity in BAL of patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*, a bacterium that has been demonstrated to produce a metallo-elastase (EDTA-inhibitable) activity (16). Nevertheless, the role of AM, particularly if activated, may be of a great importance. The AM appears to be able to internalize the PMN elastase (17), and a slow release of active enzyme from this reservoir may be one of the mechanisms contributing to the modulation of proteolysis (18).

Table 4. Effect of nosocomial infections on albumin, proteinase inhibitors (α 1 Pi, α 2M), free elastolytic specific activity (NPE/albumin), and NPE/ 2α 1 Pi ratio in bronchoalveolar lavage (BAL); values obtained from control infants (14 samples) are compared to those obtained from BPD infants when infected (11 samples) or not (35 samples)

	Control	BPD	
		Noninfected	Infected
(n of BAL samples)	(14)	(35)	(11)
Albumin (mg/L)			
Median	32	46	52*
Range	1.4–67	9–200	22–462
α 1 Pi (μ g/mg albumin)			
Median	90	62	56†
Range	50–360	16–237	5–82
α 2 M (μ g/mg albumin)			
Median	61	67	82
Range	12–357	5–212	5–160
NPE/mg albumin			
Median	30	47	59†
Range	10–110	7–236	18–215
NPE/ 2α 1 Pi			
Median	0.18	0.35†§	1.25‡
Range	0.04–0.6	0.04–2.4	0.14–6.1

p versus control values, * <0.05, † <0.01, ‡ <0.001.

§ p versus infected values, <0.01.

In agreement with the results of Ogden *et al.* (2), the increasing free elastolytic activity we found in BPD infants was not associated with increasing levels of proteinase-inhibitors. Moreover, the BAL α 1Pi/albumin ratio is significantly decreased in BPD infants as compared to controls. Two pieces of evidence support the hypothesis of local destruction and/or inactivation of α 1Pi. First, no decrease but rather an increase of serum α 1Pi, and α 2M was found in BPD infants as compared to controls. These results are in agreement with those of Ogden *et al.* (2) and suggest that very low birth wt infants are able to increase production of acute phase proteins during pulmonary inflammation. Second, the inability of BPD infants to increase BAL α 1Pi is not explained by low transudation rate. Indeed the serum α 1Pi level clearly modulates lung level of α 1Pi, a 50 000 D protein, and it is well known that increased pulmonary capillary permeability in BPD facilitates access of serum proteins to the alveoli (19). This is clearly demonstrated in our study by increased levels of BAL albumin in BPD infants and also by the detection of significant levels of BAL α 2M, a 725 000 D protein, which is normally not present in healthy adult lung (20). In contrast significant levels of α 2M have been detected in adult inflamed lung (21) and also in newborn lung during prolonged MV (22). The low antigenic level of BAL α 1Pi we found can be explained by oxidative and/or proteolytic destruction of the protein. Indeed, simple oxidative inactivation of α 1Pi by a reactive oxygen metabolite generated by PMN has been demonstrated (23). Inactivation of α 1Pi is explained by oxidation of an active site methionine residue (23), which renders this protein susceptible to proteolytic digestion (24).

Inactivated oxidized α 1Pi and a proteolytic product of degradation have been found in the early stage of BPD (1) and also in cystic fibrosis (25). As oxidation of α 1Pi reduces the binding capacity for PMN elastase 2000-fold (26), and because we measured only antigenic levels of α 1Pi, detecting both native and oxidized α 1Pi as well as α 1Pi bound to elastase, we have probably underestimated the imbalance between elastolytic activity and active α 1Pi.

Our results also emphasize the role of nosocomial infection in the course of BPD. A dramatic increase in elastase-proteinase inhibitor imbalance was shown in a subgroup of infected BPD lavages. These results support those of other authors, especially in cystic fibrosis (9, 25). Chronic infection of the lung in cystic fibrosis, with bacteria such as *Staphylococcus aureus* and *P. aeruginosa*, has been shown to participate in proteolysis of lung connective tissue (9, 25), and in such cases the mechanism of elastase-proteinase inhibitor imbalance has been shown to be the same as discussed above (26). The well-known role of acute

Table 5. Sputum bacteriology, sp act (NPE/albumin ratio), and inhibition characteristics of elastase in 11 BAL samples from infected BPD infants*

Sample	Predominant organism	Sp elastase act (NPE/mg albumin)	% Inhibition	
			PMSF	EDTA
1	<i>Staphylococcus aureus</i>	18	NA	NA
2	<i>S. aureus</i>	58	4	28
3	<i>Pseudomonas aeruginosa</i>	104	75	0
4	<i>S. aureus</i>	119	83	2
5	Enterobacter	199	56	5
6	<i>P. aeruginosa</i>	59	59	0
7	<i>P. aeruginosa</i>	127	64	32
8	Enterobacter	42	28	0
9	<i>S. aureus</i>	215	69	100
10	<i>S. aureus</i>	57	52	0
11	Enterobacter	35	11	45
Total		Median	58	3.5†
		Range	4–83	0–100

* PMSF, phenylmethylsulfonyl fluoride; NA, not assayed. † p < 0.05 between EDTA and PMSF inhibition.

nosocomial infection in the progression of BPD must be explained by increase in proteolytic destruction of the lung tissue.

Our study suggests an important role for elastase-proteinase inhibitor imbalance in the prolonged course of BPD. The development of a routine assay (27) for detection of such an imbalance in serum and/or BAL could help in the management of BPD infants, especially in cases of nosocomial infection.

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