Alveolar Macrophage Status in Bronchopulmonary Dysplasia

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ABSTRACT. The predominant inflammatory cell type within the alveolar structure in bronchopulmonary dysplasia (BPD) is the alveolar macrophage (AM). AM ability to release hydrogen peroxide, a way to evaluate the cell status, was studied in nine infants who developed clinical and radiological evidence of BPD, and was compared to those from infants without lung parenchymal disorders ($n = 6$). AM were collected by bronchoalveolar lavage which was done after the mechanical ventilation stage in the BPD group. The experiments were performed on unstimulated AM and on AM stimulated by phorbol myristate acetate. Results revealed that the amount of hydrogen peroxide accumulated in the culture medium was significantly enhanced in the BPD group, in both experimental conditions ($p < 0.01$ and < 0.001 , respectively). Furthermore, improvement of patients treated with glucocorticoids was closely related to a reduction of the alveolitis with a decrease of AM ability to generate hydrogen peroxide. These data indicate that AM activation is a central component of alveolitis in BPD and that extracellular production of oxidants by stimulated AM may play a critical role in the pathogenesis of the disease. (*Pediatr Res* 23: 470-473, 1988)

Abbreviations

AM, alveolar macrophage
BPD, bronchopulmonary dysplasia
RDS, respiratory distress syndrome
 H_2O_2 , hydrogen peroxide
PMA, phorbol myristate acetate
L, lymphocyte
PMN, polymorphonuclear
FRC, functional residual capacity
CL_{dyn}, dynamic lung compliance
RL, total pulmonary resistance
PRS, phenol red assay solution
HRP, horseradish peroxidase
BAL, bronchoalveolar lavage
FiO₂, fractional inspired oxygen
PRS, phenol red assay solution

According to numerous studies on the pathology of BPD, diffuse alveolar damage appears to be an early event in the development of this chronic lung disease, and histological descriptions have revealed a chronological progression from an

initial exudative stage of the alveolar lesion to a regenerative stage with repair (1-3). Furthermore, recent observations support the view that parenchymal inflammation could play a definite role in the development of BPD (4, 5).

The predominant inflammatory cell type within the alveolus is the AM that functions not only as a phagocytic cell but also as a secretory cell (6, 7). Among the numerous secretory products of AM that modulate the inflammatory and immune responses are the reactive O₂ metabolites. Generation of these species is associated with an increase in O₂ consumption and glucose catabolism via the hexose monophosphate shunt. These reactions are referred to as "the respiratory burst," and can be induced by a wide variety of stimuli (6-11). Furthermore, it has become increasingly apparent that AM can function as an initiator of the inflammatory response and can use "defense-like mechanisms" to contribute to lung injury. During these processes that lead to lung damage, the increased amount of O₂ metabolites production by AM is associated with a change of the cell status and is related to the concept of macrophage activation (12, 13).

AM can be collected by BAL. This procedure has proved to be safe and well tolerated in infants and children, and therefore appears to be an efficient and powerful technique for the study of pulmonary conditions in pediatrics (14). Using this new method of investigation, our study was undertaken with two objectives: 1) to investigate the capacity of AM from BPD patients to generate O₂ metabolites and therefore to evaluate the activity of cells that might contribute to lung damage; and 2) to determine whether glucocorticoids influence AM activation and therefore the inflammatory alveolar process of this chronic lung disease.

MATERIALS AND METHODS

Patients. Nine infants who developed BPD were enrolled in this study. Criteria for patients selection were as follows: 1) they had been admitted in a neonatal intensive care unit and had assisted ventilation for RDS; RDS was diagnosed on the basis of cyanosis, retraction, respiratory grunting, and radiographic findings of a reticular granular pattern and air bronchograms; 2) they developed severe BPD. The diagnosis of BPD was made as infants fulfilled all of the following criteria (15): required intermittent positive pressure ventilation during the first week of life and for a minimum of 3 days, required supplemental oxygen for more than 28 days, and developed chest x-ray changes consistent with the diagnosis of BPD. The records of these infants were evaluated for birth weight, gestational age, duration of mechanical ventilation, and duration of FiO₂ > 0.4 (Table 1). At the time of scheduled BAL, each infant had had physical and radiological evaluations, and arterialized capillary blood gases determination during air breathing (16). The pulmonary function tests performed in five patients included measurements of FRC using a spiographic system adapted to the age of the children, and of CL_{dyn} and RL by using the esophageal balloon technique (17). CL_{dyn} and RL were calculated on the basis of 10 successive

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Table 1. *Characteristics of infants with BPD*

Patient	1	2	3	4	5	6	7	8	9
Birth wt (g)	1000	2800	1200	3410	2150	2100	1930	1130	995
Gestational age (wk)	31	35	28	39	37	33	34	29	28
Ventilation duration (days)	8	120	106	7	38	39	101	87	53
FiO ₂ 0.4 (days)	2	60	57	4	10	13	85	48	39
At the time of the initial study									
Age (mo)	5	5	4	2	3	3	5	4	4
Body wt (kg)	3.4	5.5	4.4	3.9	3.5	3.5	4.6	3.2	3.7
PaO ₂ (mm Hg)	44	60	55	58	55	47	49	42	50
PaCO ₂ (mm Hg)	57	42	48	42	45	43	46	48	50

breaths at resting and expiratory level and resting respiratory frequency. FRC and mechanical parameters were expressed as percent of predicted values.

The control group included six infants without neonatal respiratory disorders. In this group, BAL was performed during screening endoscopy in an evaluation of stridor. None had an history of acute lung disease during the 4 wk preceding the BAL. At the time of the study, the mean age of the group was 6 months (± 1 month); and all these infants had normal physical development, chest radiograph, and arterial blood gases.

Technique and treatment of BAL. Written informed consent was obtained from the parents of each infant. The study was approved by the Human Experimentation Committee of St. Antoine University Hospital of Paris. The BAL was done during bronchoscopy under local anesthesia (14). The bronchoscope was introduced into a lower right lobe segment. The volume of normal saline (0.15 N NaCl) used was equivalent to 10% of the predicted FRC. The sterile solution was injected in six aliquots. Only the last five aliquots of aspiration fluids were collected. One sample of the recovered BAL fluid was used for microbiological and cytological studies including total cell count and differential cell count after a cytocentrifuge preparation.

AM isolation. AM were isolated by differential adherence (18). The BAL cells were collected by centrifugation at $400 \times g$ for 10 min at 4° C. The cells were then washed once with RPMI 1640 medium containing 30 mM NaHCO₃ and 25 mM HEPES neutralized to pH 7.4. Medium was supplemented with 50 U penicillin G and 50 μ g streptomycin per ml of medium. The washed cells were suspended at a concentration of $3\text{--}5.10^5$ cells/ml in RPMI medium and added to 35-mm Petri dishes (Falcon, Oxnard, CA). The dishes were incubated at 37° C in 5% CO₂ to allow adherence of macrophages. After 2 h of incubation, the nonadherent cells were discarded and the monolayer, which was more than 95% macrophages as determined by differential staining, was washed with Hanks' balanced salt solution.

Assay for H₂O₂ production. Production of H₂O₂ by AM was measured using the method described by Pick and Keisari (19), based on the H₂O₂-mediated and HRP-dependent oxidation of phenol red. The PRS contained 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red, and 8.5 U/ml HRP. The adherent cells were covered with 1 ml of PRS prewarmed to 37° C. AM were stimulated by the addition of 5 μ g/ml of PMA in some experimental conditions, and the dishes were incubated for 30 min at 37° C. At the end of the incubation, the culture fluids were collected and centrifuged. The cell-free supernatants were made alkaline by the addition of 10 μ l 1 N NaOH; the absorbance was determined at 610 nm against a blank of PRS to which 10 μ l of 1 N NaOH was added (Perkin Elmer Corp., Pomona, CA, Lambda 1 spectrophotometer C 632.0001). Standard curves were made, using the same preparation of PRS, with 1–30 μ M H₂O₂ solutions. The standards were treated in the same manner as the experimental cultures. In some instances, 1250 U/ml catalase was used specifically to inhibit H₂O₂ accumulation. The amount of H₂O₂ produced was calculated from the standard curve. The cells were scraped in 0.8 N NaOH for protein determination according to the method of Lowry *et al.* (20). The results were expressed as nmol of H₂O₂ generated per mg cell protein.

Reagents and media. PMA, Phenol red, HPR (type II salt free power), and catalase (bovine liver, 11,000 U/mg) were purchased from Sigma Chemical Co. St. Louis, MO. RPMI 1640 was obtained from GIBCO, Grand Island, NY. PMA was dissolved in dimethylsulfoxide at 5 mg/ml, aliquoted, and stored at -70° C.

Data analysis. Data are expressed as mean values $\pm$ SEM. Statistical significance was evaluated by unpaired *t* tests.

RESULTS

BPD patients. At the time of the study, the age of the infants ranged from 2 to 5 months (mean age 4 months). They were free of infectious disease but clinical signs of respiratory distress, mainly tachypnea, were present. The chest radiographs showed severe BPD, characterized by areas of cystic radiolucency alternating with strands of radiodensity. Arterialized capillary blood gases showed that PaO₂ was decreased to values outside the normal range (mean $\pm$ SEM 52 ± 2 mm Hg). All the patients had supplemental O₂ therapy when the BAL was scheduled. The pulmonary functions tests performed in five patients (nos. 2, 4, 7, 8, 9) indicated that FRC was not significantly different from the predicted values. In all infants, CL_{dyn} was less than -2 SD from the regression line on the diagram of predicted values of -57 , -65 , -55 , -57 , and -44% , respectively, and RL was significantly increased (more than $+2$ SD from the regression line) in each case ($+281$, $+283$, $+72$, $+175$, and $+268\%$, respectively).

Characteristics of BAL fluids. The recovery of lavage fluid ranged between 60–70% and did not correlate with the status of the infants at the time of the study. The cytological studies were reported in Table 2. The total number of cells/ml was significantly increased in the BPD group, $p < 0.05$. No marked differences in the percentages of AM, L, and PMN were apparent between the two groups. In each case, the microbiological studies of lavage fluid were negative.

O₂ metabolites release by AM. These experiments were done on unstimulated and stimulated AM after a 2 h culture duration in medium without serum. In previous studies performed in our laboratory, we have indicated that the amount of O₂ metabolites release by unstimulated and stimulated AM was linear with time up to 90 min. We have also reported that for PMA stimulation, a plateau was reached at a concentration of 5 μ g/ml (21). Herein, H₂O₂ accumulation in the medium was measured after a 30-min incubation period, and the experimental protocol included non-stimulated AM and AM stimulated by 5 μ g/ml PMA.

The results of the experiments are reported in Figure 1. The ability of AM to generate O₂ metabolites was greatly enhanced in the BPD group. A statistically significant increase of H₂O₂ release per mg cell protein was observed in the BPD group for both unstimulated AM and PMA-stimulated AM (control group *versus* BPD group, mean $\pm$ SEM), protein amount per Petri dish: 98 ± 15 mg *versus* 104 ± 17 mg; H₂O₂ release by unstimulated AM: 10.8 ± 2.1 *versus* 19.6 ± 1.7 nmol/mg protein/30 min, $p < 0.01$; H₂O₂ release by PMA-stimulated AM: 24.6 ± 1.8 *versus* 62.4 ± 4.4 nmol/mg protein/30 min, $p < 0.001$. The addition of catalase (1250 U/ml) to cultures of macrophages in PRS

without stimulant or in the presence of PMA completely abolished H_2O_2 generation.

Based on these results and supported by data from other studies, an antiinflammatory treatment was then proposed. As some parents were reluctant to give consent, prednisone treatment was given to five children (nos. 2, 3, 7, 8, 9). The therapeutic protocol was the following: 1) 2 mg/kg/day of prednisone for 1 month and then a progressive decrease; 2) a clinical, functional, and BAL evaluation 2 months after the onset of the treatment. No complications were reported. Clinical improvement in the treated group was assessed by physical examination that showed that respiratory frequency was markedly decreased. Results of functional and BAL studies after prednisone treatment are indicated in Table 3. In each case hypoxemia was corrected and CLdyn improved and was within the normal range in two infants. The results of BAL cytological studies in this treated group indicated that the total cell number/ml was significantly de-

creased with no change in the percent of various cells. The measurements of H_2O_2 generation by AM after prednisone showed a decrease of this production both in unstimulated and PMA-stimulated AM. However, at the time of the study, the amount of H_2O_2 release after PMA stimulation remained above the range observed in the control group. Further pulmonary function tests and BAL studies were not carried out in infants who received no prednisone. However, 2 months after the pulmonary evaluation, clinical conditions were not markedly changed and they still required O_2 therapy.

DISCUSSION

The pathogenesis of BPD is complex but it is widely believed that oxidant injury plays a critical role in the development of this chronic lung disease (1, 22). In BPD, oxidants, such as oxygen metabolites and other free radicals, can be generated from many sources. Extracellular formation may be initiated by stimulated phagocytes such as AM and PMN; excessive intracellular production is induced by hyperoxia (23, 24, 33).

Recently, considerable attention has been directed to the involvement of oxidants in the inflammatory process (25, 33). The pulmonary inflammation noticed during the course of the neonatal RDS has been investigated by Merritt *et al.* (5) who reported a predominance of PMN in tracheal aspirates from newborns with RDS at the early stage of the disease, whereas after the first week of life the predominant inflammatory cell type was AM. Recently, Ogden *et al.* (4), using a modified BAL procedure, showed that significant PMN and AM influxes were present at 96 h in RDS, and that PMN counts remained significantly elevated after 5 wk in BPD. It is likely that the cell population within the alveolar structure is modified during the course of the disease. Herein BAL was performed after ventilatory therapy and therefore at a later stage than the work reported above. In our population, the main feature of the BAL cytological evaluation was an increase of the total cell count/ml in the BPD group, but no differences in the percentage of AM, L, PMN were found when the two groups were compared. These observations support the consideration that at this stage of the disease, the alveolitis in BPD is mainly associated with an influx of AM, and led us to investigate the oxidative metabolism and state of activation of AM.

Herein we report evidence that AM from infants with BPD have a higher capacity to generate O_2 metabolites *in vitro* than those from control subjects. The increased H_2O_2 production was demonstrated for both stimulated and unstimulated cells. These results are similar to the data reported in adult inflammatory lung diseases such as sarcoidosis (26) and in children with interstitial lung diseases (21). The mechanisms by which these species may play a role in lung injury have been intensively investigated and many possibilities have been proposed. O_2 products can damage cells directly by oxidative attack on cell constituents including protein, lipids, and nucleic acids which could lead to cell death (24, 27). Oxidants also trigger other reactions that can

Table 2. Cytological characteristics of BAL (mean $\pm$ SEM)

Group	Cell count (10^3 /ml)	AM (%)	L (%)	PMN (%)
Control	295 ± 68	90.9 ± 2.1	7.8 ± 1.8	1.3 ± 0.5
BPD	791 ± 234	93.1 ± 2.4	4.7 ± 1.7	1.9 ± 1.1

Fig. 1. H_2O_2 release by AM from control infants and from infants with BPD. Experiments were performed on nonstimulated cells (ns) and on PMA-stimulated cells (PMA). Results are expressed as nmol of H_2O_2 released per mg protein per 30 min. The bars represent the mean $\pm$ SEM.

Table 3. Results of steroid therapy

Patient	2		3		7		8		9	
	-*	+*	-	+	-	+	-	+	-	+
PaO ₂ (mm Hg)	60	69	55	67	49	69	42	71	50	74
RL (%)	+281	+246			+72	+108	+176	+90	+268	+159
CLDyn (%)	-57	-18			-55	-11	-57	-27	-44	-39
Total BAL cells (10^3 /ml)	1310	450	2000	160	600	370	440	250	490	250
H_2O_2 generation by AM†										
Unstimulated	21.4	7.4	27.5	14.8	14.4		17	5.4	17.5	4
PMA stimulated	46.9	11.8	62.4	42.8	67.4	36.1	80.5	36.4	65.7	39.9

* Data are indicated before steroid treatment (-) and after steroid treatment (+).

† Data are expressed as nmol of H_2O_2 /mg protein after a 30-min incubation period.

indirectly amplify lung damage. These reactions involve inactivation of antiproteases, production of compounds that recruit and/or stimulate phagocytic cells to release O_2 metabolites, and interactions with arachidonic acids that generate cyclooxygenase and lipoxygenase products (24, 25). The pulmonary damage induced by these factors includes attack of the alveolar cells, alteration of surfactant and formation of atelectasis, pulmonary hypertension and increased capillary permeability, and lung infection due to altered tracheobronchial clearance and AM function (28, 29, 33).

Our data support the consideration that macrophage activation is an important component of the alveolitis observed in BPD. Based on the results herein and of other investigations, antiinflammatory treatment was started in five patients. Glucocorticoids are used extensively in the management of inflammatory disorders. In addition, it has been shown that in children with interstitial lung disease, the dramatic improvement induced by steroid treatment was associated with a remarkable reduction of the AM ability to generate H_2O_2 *in vitro* (21). The conclusions that can be drawn from the prednisone therapy in our BPD population are preliminary because this study was not double-blind and randomized. Nevertheless, the clinical and functional results are interesting. Whereas the untreated patients still required O_2 , the steroid-treated patients corrected their hypoxemia within 2 months after the start of prednisone. This improvement was closely related to a reduction of the alveolitis assessed by a decrease of the total cell number per ml in the BAL fluid and a decrease of the ability of AM to generate H_2O_2 . Several studies using corticosteroid therapy in BPD have been reported with various results, but in most of them the treatment was used in the early stages of the disease, before weaning from the respiratory support (30, 31). Herein the patients were investigated after mechanical ventilation was discontinued. When the infants were enrolled, they had severe BPD, and the steroid treatment was proposed based on classical investigations (clinical and functional), and on cytological evidence of active disease. Using these parameters, we have shown a remarkable improvement of the patient status; nevertheless 2 months after the onset of prednisone, some functional components remained abnormal and the alveolitis had not completely regressed. These results are encouraging and require further investigations to evaluate the most appropriate antiinflammatory therapeutic strategy.

In summary, these results support the consideration that alveolitis remains persistent in severe BPD, after the mechanical ventilation stage. The study of BAL cells lead to the conclusion that macrophage activation is a central component of this alveolitis, and that O_2 metabolites generation by phagocytic cells is greatly enhanced in BPD. Consequently, these data emphasize the potential involvement of oxidant injury in the pathogenesis of the disease. Based on this consideration, they provide additional interest and support for antiinflammatory treatments that might be most safely used after mechanical ventilation has been discontinued. These data also suggest that new therapeutic strategies with attempts to increase pulmonary antioxidant defenses might be of value in the management of BPD (32, 33).

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