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Cellular and Noncellular Components of Bronchoalveolar Lavage Fluid in HIV-1-Infected Children With Radiological Evidence of Interstitial Lung Damage

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Summary. Children with acquired immune deficiency syndrome (AIDS) commonly have recurrent infectious and noninfectious lung complications that ultimately end in death. To study the intensity of alveolar inflammation and to evaluate the clinical utility of bronchoalveolar lavage (BAL) in children with HIV-1 infections, we retrospectively analyzed differential cell counts, lymphocyte subsets, and fibronectin and hyaluronic acid concentrations in BAL fluid of 18 HIV-1-positive children (9 boys, mean age 3.5 years, range 5 months–8 years) with radiological evidence of interstitial lung disease, and 19 control children who had undergone BAL for clinical indications not involving the lung parenchyma (13 boys, mean age 3 years, range 2 months–14 years).

BAL fluid from 89% of the HIV-1 infected children showed CD8+ve lymphocytic alveolitis expressing HLA-DR, CD54, and CD 69 antigens. BAL fluid from HIV-infected patients typically contained markedly increased percentages and numbers of lymphocytes ($P < 0.0001$) and eosinophils ($P < 0.04$) and significantly higher concentrations of albumin ($P < 0.05$) and fibronectin ($P < 0.0006$) than fluids from control children. Whereas BAL cellular components did not differ in *P. carinii*-positive and *P. carinii*-negative HIV-1-infected children, fibronectin concentrations were significantly higher in *P. carinii*-positive than negative children. BAL cell differentials and noncellular components were related neither to severity of disease nor to patients' disease progression.

These findings indicate that BAL is useful in studying the intensity of lung inflammation in children with HIV-1 infections and radiologically documented interstitial lung disease, but provides no information on the subsequent clinical course. **Pediatr Pulmonol. 2001; 31:205–213.**

Key words: bronchoalveolar lavage; cellular and noncellular components; AIDS; children; human immunodeficiency virus; interstitial lung disease.

INTRODUCTION

Children with AIDS commonly have infectious and noninfectious pulmonary complications, which ultimately end in death.[1] Among the most frequent complications are acute pneumonia secondary to bacterial, fungal or *Pneumocystis carinii* infections, and lymphocytic interstitial pneumonitis.[1] Flexible fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) is a safe and effective method for diagnosing pulmonary infections in immunocompromised children.[2,3]

Whereas BAL cellular results (lymphocytic or neutrophilic alveolitis) have a relatively low diagnostic yield in adults with AIDS-related pulmonary disease and in children,[4–9] BAL differential cell counts can reliably predict the outcome of patients with human immunode-

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TABLE 1—Clinical Features of Patients[1]

Patients	Gender	Age (years/months)	CDC class before BAL	BAL culture	Months of follow-up	CDC class after follow-up
1	F	0.5	C2	Pc	4	Died
2	M	1.2	B2	Pc	51	C3
3	M	1.2	A1	Sterile	82	B2
4	M	1.2	C3	Pc	33	Dropout
5	F	1.3	A1	Pc	47	C2
6	F	1.4	C3	Pc	12	C3
7	F	1.7	B2	Pc	50	C3
8	F	1.9	A1	Sterile	17	A1
9	M	2.9	C3	Sterile	50	C3
10	F	3.1	C3	Pc	37	C3
11	F	4.2	C3	*Candida*	1	Died
12	M	4.5	C3	Pc	17	C3
13	F	5.0	B3	Sterile	37	C3
14	M	5.6	B3	Pc	51	C3
15	M	6.0	B2	Sterile	50	B2
16	F	6.0	C2	Pc	36	C2
17	M	7.1	B3	Sterile	15	B3
18	M	7.8	B2	Pc	18	C2

[1]Pc, *Pneumocystis carinii*; BAL, bronchoalveolar lavage; CDC, Center for Disease Control.

ficiency virus type 1 (HIV-1) infections and pulmonary involvement.[10–13] For example, the finding of BAL neutrophilia in HIV-1-infected patients with opportunistic infections correlates strongly with an increased risk of death.[14] The combined analysis of cellular and noncellular components recovered from BAL could help to explain the role of HIV-1 in the pathogenesis of AIDS-related pulmonary manifestations. The progressive decline in lung function could result from secondary infections due to pulmonary immunologic defects, or could be secondary to the immunological response to retrovirus infection.[15–16]

To evaluate the intensity of alveolar inflammation and the clinical usefulness of BAL profiles in children with HIV-1 infection and radiologically documented interstitial lung damage, we determined BAL differential cell counts, lymphocyte subsets, and fibronectin and hyaluronic acid concentrations in these patients and in controls.

PATIENTS AND METHODS

Patients

From February 1993 to May 1997, 18 consecutive HIV-infected children (9 boys and 9 girls; mean age, 3.5 years; range, 5 months–8 years) underwent flexible bronchoscopy and bronchoalveolar the lavage at the Pediatric Department IV, University of Rome ''La Sapienza.'' The diagnosis of AIDS was made according to criteria established by the Center for Disease Control.[17] In all 18 cases, HIV-1 was transmitted vertically from infected mothers to their offspring. Before undergoing flexible fiberoptic bronchoscopy and BAL, all children were clinically classified according to the Center for Disease Control guidelines for pediatric HIV infection (clinical and immunological status) on a severity scale ranging from A1 to C3.[17] All children had a recent chest roentgenogram (1–7 days) showing diffuse nodular or reticulonodular lung patterns, according to McLoud et al.,[18] and suggestive of interstitial lung damage. Six children also had radiological evidence of lung infiltrates. The indication for bronchoscopy and BAL was deterioration in their respiratory status, manifested by increasing tachypnea and fever. Children were followed for a mean of 2.8 years (range, 1 month–6.8 years) and underwent a second staging procedure at the end of the follow-up (Table 1). In 13/18 examinations (72%), BAL fluids gave diagnostic information. *Pneumocystis carinii* was detected in 11 procedures (61%), *Candida* in 1, and *Streptococcus pneumoniae* in 1 patient (Table 1).

ABBREVATIONS

AIDS	Acquired immune deficiency syndrome
BAL	Bronchoalveolar lavage
ELISA	Enzyme-linked immunoadsorbent assay
FACS	Fluorescence-activated cell sorter
HIV-1	Human immunodeficiency virus type 1
HLA-DR	Human leukocyte antigen-D region
PBS	Phosphate-buffered saline
SaO_2	Arterial oxygen saturation

Controls

The control group consisted of 19 children (13 boys and 6 girls; mean age, 3 years; range, 2 months–14 years) undergoing fiberoptic bronchoscopy for various clinical indications, such as persistent stridor (n = 10 cases); follow-up evaluation 2 months after the removal of a foreign body (n = 4); investigation of chronic cough resistant to medications (n = 3); assessing progression of

left lobar emphysema associated with mild stenosis of the lingular bronchus 2 years after the initial diagnosis (n = 1); and reassessing the stenosis of the main left bronchus 1 year after the initial diagnosis (n = 1).

Parents of patients and controls provided written informed consent, and the procedures had the approval of the Hospital Ethics Committee.

Procedure

The procedures used for flexible bronchoscopy, BAL, and lavage fluid analysis have been described elsewhere.[19] Before bronchoscopy, children received topical lidocaine anesthesia of the upper airways and intravenous sedation with meperidine 1–2 mg/kg. A Pentax FB 10H fiberoptic bronchoscope (Tokyo, Japan) was used in children younger than 6 years, and a Pentax FB 15H (Tokyo, Japan) in children older than 6 years. During fiberoptic bronchoscopy, children breathed spontaneously around the bronchoscope and received supplemental oxygen through a nasal catheter. Children were monitored during the procedure for heart rate and arterial oxygen saturation (SaO_2) with an Ohmeda pulse oximeter. BAL fluid was obtained from the right middle lobe or lingula by instilling three 10-mL aliquots (in children under 6 years) or three 20-mL aliquots (in children over 6 years) of prewarmed sterile saline via the suction channel of the bronchoscope. Each aliquot was immediately suctioned back into the same syringe and subsequently stored in ice. The first aliquot was used for microbiological studies. The second and third aliquots were pooled and used for cellular and noncellular analyses. Differential cell counts were determined by two independent observers on May-Grünwald-Giemsa-stained cytospin preparations (10^5 cells/0.5mL; spun at 500*g*). At least 200 cells were studied. BAL fluid was centrifuged at 500*g* for 10 min, and the supernatant was removed and stored at $-70°C$. Cell pellets were resuspended in phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide.

Analysis of Lymphocyte Subsets

Lymphocyte subsets in blood and BAL fluid were analyzed in 9 patients according to standard methods recommended by the Becton-Dickinson Monoclonal Center (Mountain View, CA). In brief, 50μL of either whole blood or BAL cells (1×10^5 cells) were incubated in the dark for 30 min with saturating concentrations of fluorescein or phycoerythrine-conjugated monoclonal antibody. Erythrocytes were lysed by adding 3 mL of lysing solution (Becton-Dickinson Immunocytometry System, San Jose, CA) for 10 min in the dark. Leukocytes were washed twice with PBS containing 2% fetal calf serum and 0.1% sodium azide, and were finally resuspended in PBS containing 2% of formaldehyde. Fixed cells were analyzed by flow cytometry with a fluorescence-activated cell sorter (FACScan), using the Lysis II program. The number of immunofluorescence-positive cells was determined in 10,000 analyzed cells. Specific binding of monoclonal antibodies was controlled by subtraction of isotype-matched mouse immunoglobulins. Monoclonal antibodies against CD3 (T cells), CD4 (T helper/inducer cells), CD8 (T suppressor/cytotoxic cells), CD19 (B cells), CD16 (natural killer cells), CD25 (α chain of the interleukin-2 receptor), HLA-DR (human leukocyte antigen-D region), adhesion molecule-ICAM 1, and CD69 (early activation antigen) were purchased from Becton-Dickinson.

Noncellular Components

The total amount of protein in BAL was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany), based on the principle of protein binding to Coomassie brilliant blue.[20] The intraassay coefficient of variation was 2.7%, the interassay coefficient was 4%, and the detection limit was 12 mg/L. Albumin was measured with the nephelometric method, using the Beckman assay protein system (Beckman Instruments, Inc., Brea, CA). The total intraassay and interassay coefficients of variation were below 3.3%, and the detection limit was 6 mg/L. Fibronectin was measured with a double sandwich enzyme-linked immunoadsorbent assay (ELISA), as described previously.[21] The intraassay and interassay variation was $< 7\%$, and the detection limit was 10 μg/L. Hyaluronic acid was measured in duplicate samples by means of a radioimmunoassay (Pharmacia Diagnostic, Uppsala, Sweden), as previously described.[22] The detection was 1 μg/L. Variability of measurements was $<10\%$.

Statistical Analysis

Data are reported as means ± SE and medians. Differences between groups were determined with the Mann-Whitney U test for two independent samples. A *P*-value ≤ 0.05 was considered statistically significant. Cutoff limits of variables were calculated from data obtained from 19 controls. Values twice the standard deviation from the mean of the corresponding control values were taken as cutoff limits. The relation between the percentage of patients exceeding the cutoff limits and the percentage of patients' outcomes within different subgroups was determined with the Chi-square test.

RESULTS

Clinical Classification

Before undergoing flexible bronchoscopy and BAL, 3 children were CDC Class A1, 4 were B2, 3 were B3, 2 were C2, and 6 were C3. At the end of follow-up, only

TABLE 2—BAL Cellular Components in HIV-Infected Patients and Controls

	Patients (n = 18)			Controls (n = 19)			
	Mean	Median	Minimum–maximum	Mean	Median	Minimum–Maximum	*P*-value
%BAL recovery	60	63.0	35–75	45.0	46	20–65	0.0006
Cells/mL ($\times 10^3$)	848	800.0	350–1450	536.0	400	200–1300	0.01
Differential cell count							
Macrophages							
%	52	48.0	18–87	86.0	87	71–98	0.0001
$\times 10^3$/mL	412	303.0	126–900	451.0	386	182–1001	0.7
Neutrophils							
%	8	2.0	0–48	5.0	3.5	0–17	0.2
$\times 10^3$/mL	76	12.0	0–672	30.0	11	0–221	0.7
Lymphocytes							
%	39	35.0	4–83	8.0	7	2–22	0.0001
$\times 10^3$/mL	348	327.0	37–972	51.0	33	6–253	0.0001
Eosinophils							
%	1	0.5	0–8	0.2	0	0–2	0.03
$\times 10^3$/mL	12	0.0	0–104	0.8	0	0–5.4	0.04

1 child was Class A1, 2 were B2, 1 was B3, 3 were C2, and 8 were C3. Three patients died during the follow-up period (Table 1).

Tolerance

No complications arose during or after fiberoptic bronchoscopy or BAL procedures. None of the children decreased their SaO_2 below normal values.

Cytological Results

The percentage of BAL fluid recovered and the total number of cells/mL were both significantly higher in HIV-infected patients than in controls ($P < 0.01$) (Table 2). Alveolar macrophage percentages were significantly lower in patients than in controls, but total macrophage numbers were similar in the two groups (Table 2; Fig. 1A, B). BAL fluids from 16 (89%) of the HIV-1-infected patients showed lymphocytic alveolitis (Fig. 1C, D). Lymphocyte subset analysis identified almost 80% of these cells as CD8 + ve (Table 3). As a group, BAL specimens from HIV-1-infected patients showed a marked increase in the number and percentage of lymphocytes ($P < 0.0001$) (Table 2). No significant differences were observed in BAL neutrophil numbers and percentages in patients and controls (Table 2). Only 3 patients had neutrophilic alveolitis (Fig. 1E, F). Finally, BAL fluids from HIV-1-infected children had significantly higher eosinophil percentages and numbers than fluids from controls (Table 2 and Fig. 1G, H), and flow cytometry identified a significantly higher percentage of suppressor/cytotoxic lymphocytes in BAL fluids than in blood. The CD4/CD8 ratio was lower in BAL fluids than in blood (0.1 vs. 0.3) (Table 3). Numerous lymphocytes in BAL fluids and blood expressed the HLA-DR antigen. A large number of BAL lymphocytes coexpressed CD54 and CD69 antigens, whereas blood lymphocytes did not (Table 3). Comparison of BAL fluid results showed no diferences in celluar components or lymphocyte subsets in *P. carinii*-positive and *P. carinii*-negative HIV-infected patients. No relations were observed between BAL cellular profiles and the patients' clinical severity and disease progression.

Noncellular Components

BAL fluid from HIV-infected patients contained significantly higher albumin ($P < 0.05$) and fibronectin ($P < 0.0006$) concentrations than BAL fluid from control children (Fig. 2). Fibronectin concentrations were significantly higher in BAL fluids from *P. carinii-positive than in fluids from P. carinii*-negative HIV-1-infected patients ($P < 0.04$; Fig. 2). No relationships were observed between BAL noncellular components and clinical severity or outcome of patients.

DISCUSSION

Our study confirms that assessing BAL cellular and noncellular components is useful in assessing the intensity of airway inflammation in children with HIV-1 infection and radiologically documented interstitial lung disease. Most of these HIV-1 infected patients had CD8 + ve cytotoxic lymphocytic alveolitis. Previous reports have shown that lymphocytic alveolitis is common in AIDS patients with and without pulmonary involvement, and our finding confirms the observations of other investigators.[5–7] The source and the role of CD8 + ve lymphocytes in these patients' lungs is poorly defined. One pathogenic mechanism proposes a local increase in CD8 + ve cells secondary to an in situ expansion of

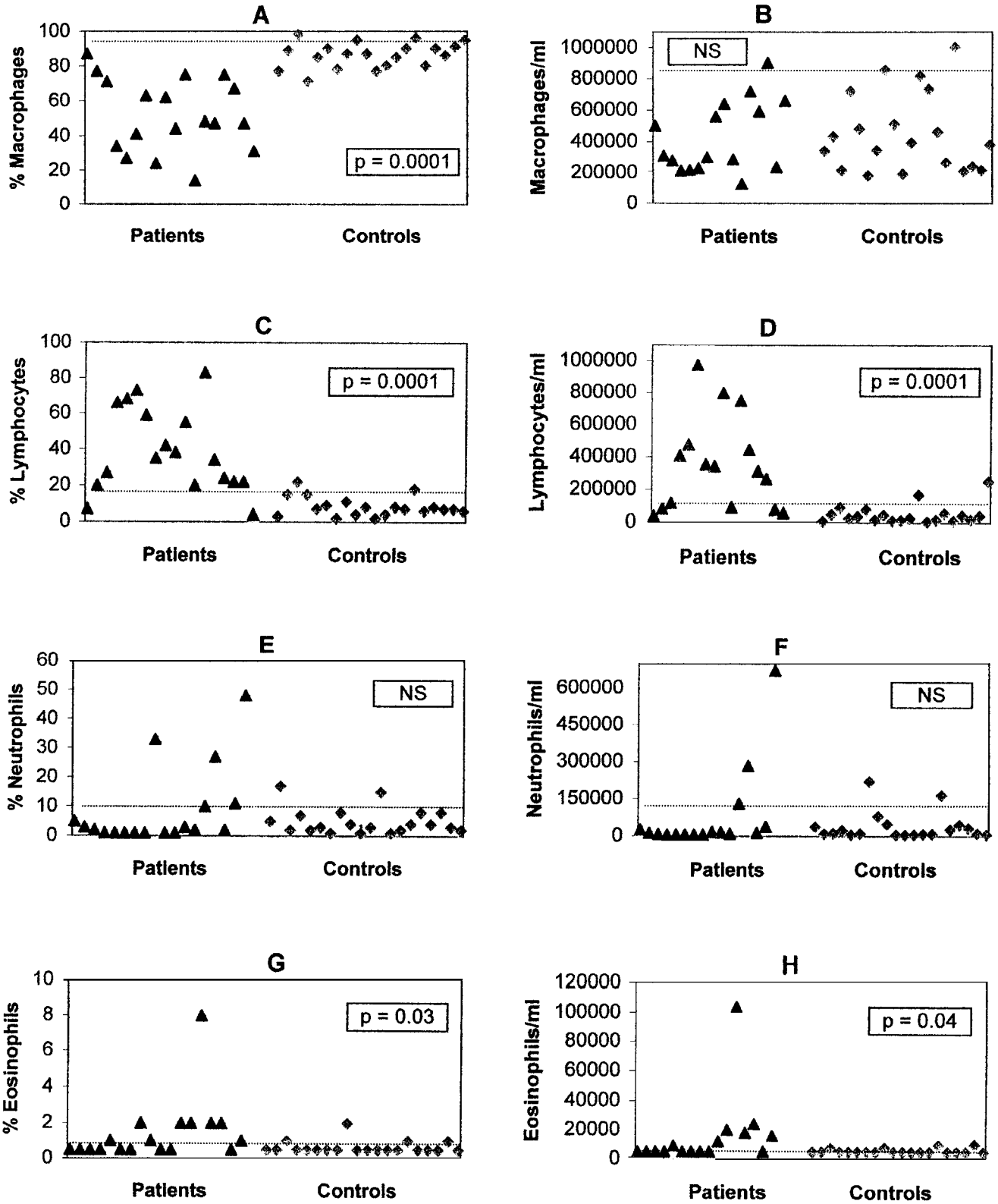

Fig. 1. BAL cell differentials in control and HIV-infected children. Ninety percent of the observations in the control group were below the stippled line (2 standard deviations above mean of control values). Designations A–H refer to text.

TABLE 3—Lymphocyte Subsets in Blood and in BAL of HIV-Infected Children With Interstitial Lung Damage

	Blood				BAL			
	n	Mean	Median	Minimum–maximum	n	Mean	Median	Minimum–maximum
CD3 (T cells)	7	69.0	68.0	46–94	9	85.0	89.0	53–92
CD19 (B cells)	5	17.0	17.0	3–41	8	2.0	2.0	0–4
CD3 CD4 (helper/inducer)	7	9.0	8.0	1–20	9	6.0	7.0	1–10
CD3 CD8 (cytotoxic/suppressor)	7	53.0	47.0	30–74	9	75.0	78.0	50–88
CD3 HLA-DR (Class II major histocompatibility molecule)	7	28.0	28.0	11–42	9	31.0	37.0	10–58
CD16 CD56 (natural killer)	7	4.0	4.0	1–9	8	2.0	1.0	0–5
CD3 CD54 (adhesion molecule, ICAM–1)	4	1.0	0.5	0–3	5	17.0	5.0	1–39
CD3 CD25 (IL–2 receptor)	4	3.0	3.0	1–4	5	1.0	1.0	0–2
CD3 CD69 (early activation antigen)	2	6.0	6.0	3–9	3	60.0	61.0	38–81
CD4/CD8 ratio	7	0.2	0.2	0.1–0.6	9	0.1	0.1	0.1

specific cytotoxic T-lymphocytes against virus-infected cells.[15] In other interstitial lung disorders, lymphocytes accumulate within the lung through an IL-2-driven proliferation of preexisting T-cells.[15] BAL T-lymphocytes from HIV-1-seropositive patients with lymphocytic alveolitis express a fully functional IL-2 receptor.[23–26] In our study, few T-lymphocytes coexpressed IL-2 receptors, whereas many expressed the HLA-DR and CD69 antigens. In particular, lymphocytes expressing the HLA-DR antigen were abundantly present in our patients' BAL fluid and blood. In contrast, lymphocytes expressing the CD69 antigen were present only in BAL fluid, suggesting that these were activated cells.

An alternative mechanism has been proposed to explain lymphocyte accumulation within the lung; it suggests recruitment of specific cytotoxic lymphocytes against virus-infected cells from the peripheral blood to the lung.[15] Interestingly, in our study few BAL T-lymphocytes coexpressed the CD54 phenotype. This finding implies that, at least in children, T-lymphocytes may be recruited from the blood and compartmentalized in the lung.

Our analysis of BAL cell differentials showed a similar number of alveolar macrophages in BAL fluids in HIV-infected patients and controls. This finding has been confirmed by others.[11] Our study in children provides no information on the state of activation of these cells, but previous reports in adult patients with HIV-1 infection described morphologic signs of activation and release a series of biologically active mediators of the immune system.[27–29] As a group, our HIV-infected children had significantly higher numbers/mL of BAL eosinophils than the control group, suggesting that these cells contribute to the intensity of lung inflammation.

Exstensive investigations have attempted to clarify the mechanisms responsible for lung damage in patients with HIV-1 infection. HIV has been detected in various lung cell types, includig lymphocytes,[15] alveolar macrophages,[30] dendritic cells,[31] and fibroblasts.[32] The immunological response to virus-infected cells can contribute to noninfectious lung damage. Although virus-specific CD8 + ve cells help to defend the lung against the HIV-1 virus, they may also injure the respiratory epithelium. The presence of CD8 + ve lymphocytes in BAL fluid correlates with increased epithelial permeability, but both abnormalities are also seen in patients without pulmonary involvement.[5,33]

To determine whether BAL noncellular components were involved in the immune-mediated lung damage in BAL fluids from our HIV-1-infected patients, we measured concentrations of two important extracellular matrix components that mediate repair processes during interstitial lung damage, i.e., fibronectin and hyaluronic acid.[34,35] Fibronectin interacts with a large number of microorganisms, and is potentially important in microbial adherence to airway epithelium and subsequent infections of the respiratory system.[36] Recent evidence shows that fibronectin plays an important role in the pathogenesis of *P. carinii* infection, mediating the attachment of the parasite to cell surfaces.[37] BAL fluids from all the HIV-1-infected children we studied contained elevated amounts of fibronectin, with significantly higher concentrations in children with *P. carinii* infection than in those without. Our in vivo findings indicate that the presence of fibronectin in these patients' BAL fluid may favor opportunistic infections commonly seen in these children. Conversely, hyaluronic acid concentrations in our patients' BAL fluids were invariably low and did not differ significantly from control values. In patients with interstitial lung disease, elevated hyaluronic acid is normally associated with a derangement of the alveolar walls and lung fibrosis.[38,39] Hence, our HIV-infected patients' low hyaluronic acid concentrations could partly explain why none of them had severe abnormalities in the alveolar structures.

We found no relation between BAL cellular components and the patients' subsequent clinical course. The clinical usefulness of studying cellular and noncellular

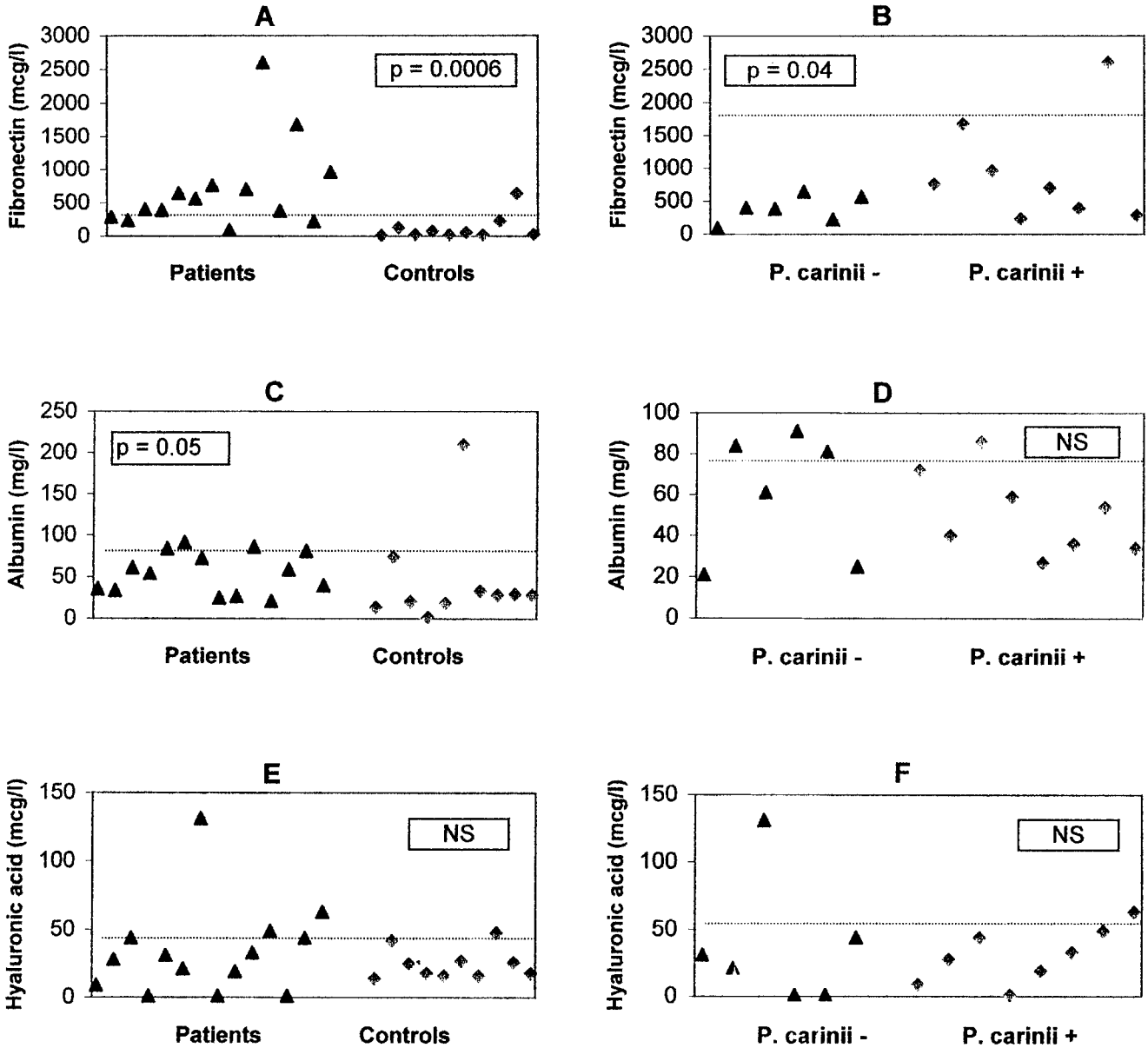

Fig. 2. BAL noncellular components in control and HIV-infected patients (A, C, E), and in *P. carinii*-negative and *P. carinii*-positive patients. (B, D, F) Ninety percent of the observations in the control group were below the stippled line (2 standard deviations above mean of control values).

components in BAL fluid from these patients remains controversial. Several reports have shown that BAL cell profiles in adult patients are related to the outcome of the disease. For example, Agostini et al.[11] reported that increased CD8 + ve lymphocytes in BAL fluid correlated significantly with an early fatal outcome of patients. Furthermore, the finding of BAL neutrophilia in HIV-1-infected patients with opportunistic infections strongly correlated with an increased risk of death.[7] In our series of HIV-1-infected children, we found no correlation between CD8 + ve cell numbers in BAL fluid and disease progression. Only 3 patients in our study had neutrophilic alveolitis. One child had a concomitant bacterial infection, a second child had a *Candida* infection and died 1 month after BAL examination, and a third child who had AIDS had a severe *Pneumocystis carinii* infection. Owing to the small number of patients, we found no significant associations between neutrophilic alveolitis and progression of disease. The 3 children with high neutrophil percentages in BAL fluids probably had more severe clinical disease than the other patients.

Several reports have described distinctive BAL profiles in AIDS patients with *P. carinii* infection. The presence of neutrophilic alveolitis is common in HIV-1-infected

patients with *P. carinii*[8] and is apparently a poor prognostic sign.[14] Furthermore, Fleury-Feith et al.[40] found that BAL eosinophilia was associated with *P. carinii* pneumonitis in AIDS patients. The BAL profiles in our patients did not distinguish *P. carinii*-positive from *P. carinii*-negative patients.

In conclusion, our findings indicate that BAL provides useful information on the intensity of lung inflammation in children with HIV-1 infections and radiologically documented interstitial lung disease, but does not help to define the subsequent clinical course.

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