BAL in Children*

A Controlled Study of Differential Cytology and Cytokine Expression Profiles by Alveolar Cells in Pediatric Sarcoidosis

Valerie Tessier, MD; Katarina Chadelat, MD; Arnelle Baculard, MD; Bruno Housset, MD; and Annick Clement, MD, PhD

Study objective: The development of BAL in children for both research and clinical purposes has been limited so far by the difficulty in establishing reference values. The aim of the study was (1) to define composition of BAL cellular components in control children and to evaluate the ability of these cells to express various cytokines, and (2) to study modifications of differential cytology and BAL cell cytokine responses in children with interstitial lung disorders.

Populations and methods: Two groups were investigated: a control group of 16 children who were concluded to be free of parenchymal lung disease after complete pulmonary investigation, and a group of 11 children with pulmonary sarcoidosis. Differential cytology was evaluated by standard techniques. BAL cell cytokine expression was studied at the level of messenger RNA (mRNA) by reverse transcription-polymerase chain reaction (RT-PCR) methods.

Results: In the control group, differential cell counts appeared to be similar to values reported in adult populations with normal distribution of the data and no influence of age. In this group, no transcripts for interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, and transforming growth factor-β (TGF-β) could be detected. In children with sarcoidosis, different profiles of IL-1β, TNF-α, IL-6, and TGF-β expression were individualized which seemed to be related to the activity and/or severity of the disease, IL-6 and TGF-β mRNA being observed only in the more severe forms.

Conclusion: These data provide information on BAL cell number and function in children. Characterization of BAL cytokine expression patterns during the course of interstitial lung diseases in children may be of great interest for evaluation of disease activity and/or severity and therefore for planning of therapy.

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Key words: bronchoalveolar lavage; childhood; cytokine; gene expression; sarcoidosis

Abbreviations: AM=alveolar macrophage; cDNA=complementary DNA; CLdyn=dynamic lung compliance; IL=interleukin; mRNA=messenger RNA; PCR=polymerase chain reaction; RT-PCR=reverse transcription-polymerase chain reaction; TGF-β=transforming growth factor-β; TLCO=lung transfer factor for carbon monoxide; TNF-α=tumor necrosis factor-α

In adults, BAL has been extensively developed since the fiberoptic bronchoscope has become available.1 It is now considered a powerful technique for recovering lung cells and proteins from patients with a variety of lung diseases. Indeed, in recent years, a growing number of studies have used BAL to investigate the pathologic processes associated with respiratory disorders in the distal airways and alveoli. By showing that the types, the numbers, and the phenotypes of cells present in the BAL fluid of patients with lung diseases differ from those present in the BAL of normal subjects, these studies have provided evidence that BAL is not only a valuable research tool but may also be of value in the diagnosis and management of respiratory diseases.

In children, BAL has proved to be safe and well tolerated, and has a considerable potential of development for both research and clinical purposes.2,3 However, this development has been limited so far by the difficulty in defining the composition of normal components of the BAL fluid in children. Indeed, for ethical reasons, cellular and noncellular BAL values to refer to cannot be obtained from normal volunteers. They can be established only from children who have undergone a bronchoscopy with lavage under various

*From the Departments of Physiology (Drs. Tessier and Clement) and Pediatric Pulmonology (Drs. Chadelat and Baculard), Hospital Trousseau, St. Antoine Medical School, University of Paris, Paris, France; and the Department of Pulmonology (Dr. Housset), CHIC Creteil, France.

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Reprint requests: Dr. Clement, Department of Physiology, Hospital Trousseau, 26 av. Dr.Netter, 75012 Paris, France

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Clinical Investigations
circumstances and who were concluded retrospectively to be free of alveolar pathologic processes, based on the results of complete pulmonary investigations.

Over a 3-year period, BAL samples were collected from children referred to the pulmonary department and who had bronchoscopy with lavage under local anesthesia. Among these patients, we were able to individualize two groups: one group that included children with no evidence of lung parenchyma disorders after complete pulmonary investigations, and a second group of patients with interstitial lung disease and for whom the diagnosis of pulmonary sarcoidosis could be established. The reason for the selection of this group is explained by the fact that, in contrast with the number of studies reported in the literature on pulmonary sarcoidosis in adult patients, sarcoidosis has been poorly investigated in children so far. However, it is likely that the progression of the disease in the pediatric population is different from the one observed in adults as it seems to run a more aggressive course. Also, in a previous study, we have observed the persistence of a high degree of alveolitis even after a long duration of steroid treatment in children with sarcoidosis.

Based on these observations, one can hypothesize that the progression of pulmonary inflammatory processes, including local cytokine responses, might differ in growing vs mature lung during the course of sarcoidosis, and that information obtained from studies performed in adults cannot be applied to the pediatric population.

Using the BAL samples, the aims of our study were (1) to define the composition of the cellular components in control children and to evaluate the ability of these cells to express various cytokines under standard conditions, and (2) to study modifications of differential cytology and of BAL cell cytokine responses in children with pulmonary sarcoidosis. In the present work, we focused on cytokines known to play an important role in pulmonary inflammatory processes. This includes interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, and TGF-β, and the study was designed to document the sequence in the expression and therefore in the involvement of these molecules during the course of the disease in a pediatric population.

**Materials and Methods**

**Study Population**

**Control Group:** From January 1992 to January 1995, we were able to include 16 children in this group (Table 1). BAL was performed during screening endoscopy in an evaluation of stridor or if inhalation of foreign body was suspected; it was also part of the pulmonary investigations programmed if sarcoidosis was suspected, mainly because of uveitis. All the patients were considered as free of lung disease retrospectively. This was established based on the results of complete pulmonary explorations. None had a history of acute lung disease during the 4 weeks preceding the BAL. None received therapy at the time of investigation nor had they within the previous month. All the patients had normal results of physical examinations and chest radiographs. Results of functional tests were within the range of predicted values for height-matched children. Microbiologic (bacteriologic and viral) analysis of the BAL fluid was sterile.

**Group of Children With Sarcoidosis:** This group included 11 children from 4 to 16 years old (mean, 12.2 years) (Table 2). The population (seven black and four white children) consisted of eight girls and three boys. The diagnosis of sarcoidosis was established on the basis of compatible clinical and radiographic findings, histologic evidence of noncaseating epithelioid cell granulomas of various tissues (eight from liver, one from lung, one from lymph node, and one from kidney), and exclusion of other granulomatous lung disorders. As previously described, the conditions of patients were staged by the traditional radiographic criteria, and pulmonary function tests were performed. Results of dynamic lung compliance (CLdyn) and lung transfer factor for CO (TLCO) are listed in Table 2. They were expressed as percent of predicted values for height-matched children, values less than 75% of predicted values being considered as significantly decreased.

After the initial assessments, all the patients were treated with steroids. The protocol of oral prednisone therapy used in the department was as previously described; 1 mg/kg/d for 6 to 8 weeks, followed by gradual tapering (1.5 mg/kg every other day for 4 weeks, and then progressive decrease guided by the disease progression to a maintenance level of 10 to 15 mg). In cases of relapse, steroid treatment was increased up to 1 mg/kg/d for 1 month and then tapered again. Data on pulmonary function tests and BAL studies at the time of the first study are given in Table 2 (left). After 6 months of follow-up, a second study with complete pulmonary investigations, including BAL studies and pulmonary function tests, was performed and results are listed in Table 2 (right); at this time, all the patients were still receiving steroids.

Informed parental consent was obtained in each case before the procedure, which was approved by the Human Experimentation Committee of Cochin University, Paris, France.

**Bronchoalveolar Lavage**

BAL was performed during bronchoscopy under local anesthesia, as previously described. Briefly, patients were premedicated with midazolam (0.3 mg/kg of body weight) and local anesthesia was achieved with topical lidocaine (2%). The bronchoscope was introduced into a lower right lobe segment. The volume of sterile normal saline solution instilled was equivalent to 10% of the functional residual capacity. The sterile solution at 37°C was injected in six aliquots, and only the last five aliquots of aspirated fluid were collected and pooled. The first portion of the BAL was not examined.

**BAL Cell Studies**

BAL fluid was centrifuged at 300 x g for 10 min at 4°C and the cell pellet was resuspended in phosphate-buffered saline solution. An aliquot was used for cytologic studies that included total cell counts determined using a hemocytometer and differential cell counts using a Wright-Giemsa-stained cytocentrifuge preparation. Another aliquot was used for microbiologic studies.

**Cytokine Expression Studies**

**RNA Extraction of BAL Cells:** RNA was extracted as previously described. The BAL cell pellet was lysed in 500 μL of guanidium isothiocyanate solution (4 M guanidium isothiocyanate, 0.1 M Tris at a pH of 7.5, 0.5% N Lauryl sarcosine, 1% β-mercaptoethanol). After addition of 10 μg of transfer RNA (tRNA) (10 mg/mL; Boehringer Mannheim Biochemicals; Mannheim, Germany), the suspension was layered over 500 μL of 5.7 mol/L CaCl2 and centrifuged for 2.5 h at 280,000 x g in an ultracentrifuge (Beckman TL-100).
**Table 1—Results of BAL Cell Counts From the 16 Children Included in the Control Group**

<table>
<thead>
<tr>
<th>Patient No./Age</th>
<th>BAL Fluid Recovery, %</th>
<th>Total Cells/mL ×10^5</th>
<th>AM, %</th>
<th>L, %</th>
<th>N, %</th>
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<tbody>
<tr>
<td>1/2 mo</td>
<td>57</td>
<td>680</td>
<td>98</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2/2 mo</td>
<td>67</td>
<td>460</td>
<td>92</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>3/2 mo</td>
<td>85</td>
<td>670</td>
<td>98</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4/4 mo</td>
<td>80</td>
<td>180</td>
<td>93</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5/6 mo</td>
<td>65</td>
<td>460</td>
<td>77</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>6/6 mo</td>
<td>68</td>
<td>240</td>
<td>92</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>7/8 mo</td>
<td>68</td>
<td>230</td>
<td>89</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>8/9 mo</td>
<td>70</td>
<td>230</td>
<td>89</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9/1 yr</td>
<td>69</td>
<td>90</td>
<td>88</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>10/2 yr</td>
<td>76</td>
<td>430</td>
<td>88</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>11/2 yr</td>
<td>67</td>
<td>530</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>12/2 yr</td>
<td>59</td>
<td>150</td>
<td>96</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>13/7 yr</td>
<td>52</td>
<td>340</td>
<td>85</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>14/7 yr</td>
<td>79</td>
<td>480</td>
<td>86</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>15/7 yr</td>
<td>61</td>
<td>270</td>
<td>94</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>16/8 yr</td>
<td>62</td>
<td>170</td>
<td>84</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

* L=lymphocytes; N=neutrophils.

The RNA pellet was resuspended in 200 μL of 1 M Tris-HCl ethylene diamine tetraacetic acid buffer (TE) 0.1% SDS solution. After phenol-choroform extraction, it was ethanol precipitated, centrifuged, dried, and resuspended in a volume of dH2O adjusted to the cell number (8 μL per 10^6 cells).

**Reverse Transcription:** RNA was reverse transcribed into complementary DNA (cDNA) after its denaturation at 65°C for 10 min, as previously described. The reverse transcription was performed at 37°C for 60 min. The 20-μL volume reaction included 8 μL of the denatured RNA, 1 μL RNase inhibitor (40 U/μL; Boehringer Mannheim Biochemicals), 1 μL of 200 mM DTT, 1 μL oligo-dT primer (0.5 μg/μL; Boehringer Mannheim Biochemicals), 4 μL reverse transcription buffer (5 X RT buffer, BRL), 2 μL of 5 mM dNTP mix (Boehringer Mannheim Biochemicals), 1 μL of moloney murine leukemia virus reverse transcriptase (MMLV-RT, 20 U/μL; Gibco-BRL), 2 μL dH2O. Reaction was stopped by incubation at 65°C for 5 min. The cDNA was then stored at -20°C.

**Amplification of cDNAs by PCR and Analysis:** Five different cDNAs were amplified: cDNAs to β-actin (actin), IL-1β, TNF-α, IL-6, and TGF-β. Amplification was performed by preparing a cDNA containing reaction mix for each patient. The PCR reaction mix consisted of 10 μL of cDNA preparation, 50 μL of 10×reaction buffer (100 mM Tris at a pH of 8.3, 500 mM KCl; Perkin Elmer-Cetus), 20 μL of 1.25 mM dNTP mix (Boehringer Mannheim Biochemicals), 1 μL of Taq polymerase (5 U/μL; Perkin Elmer-Cetus), and 399 μL dH2O. This mix was divided into five aliquots of 96 μL each, and 4 μL of oligonucleotide 5’ and 3’ sequence-specific primers (50 pmol each) were added. Nucleotide sequences for oligonucleotide 5’ and 3’ primers used in the PCR reaction are listed in Table 3. Amplification was carried out in a PCR thermocycler (Perkin Elmer-Cetus), with the following program: denaturation at 92°C for 1 min, reannealing for 1 min at 55°C, and primer extension at 72°C for 2 min. A 30-μL aliquot of the PCR reaction mixture was analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. Comparison to the molecular weight standards confirmed the predicted sizes for amplified products (Table 3). Results were expressed qualitatively as presence or absence of transcripts for a given cytokine.

**Southern Blot Hybridization:** To further confirm the specificity of the amplification products, hybridization with a nested oligonucleotide was performed.

**Table 2—Characteristics of Patients With Sarcoidosis**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Total Cells/mL ×10^3</th>
<th>AM, %</th>
<th>L, %</th>
<th>N, %</th>
<th>CLdyn, %</th>
<th>TLCO, %</th>
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<tbody>
<tr>
<td>1</td>
<td>220</td>
<td>60</td>
<td>36</td>
<td>4</td>
<td>94</td>
<td>64</td>
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<tr>
<td>2</td>
<td>240</td>
<td>48</td>
<td>51</td>
<td>1</td>
<td>68</td>
<td>54</td>
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<tr>
<td>3</td>
<td>400</td>
<td>78</td>
<td>21</td>
<td>1</td>
<td>62</td>
<td>89</td>
</tr>
<tr>
<td>4</td>
<td>760</td>
<td>95</td>
<td>4</td>
<td>1</td>
<td>85</td>
<td>78</td>
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<tr>
<td>5</td>
<td>180</td>
<td>63</td>
<td>36</td>
<td>1</td>
<td>65</td>
<td>71</td>
</tr>
<tr>
<td>6</td>
<td>400</td>
<td>48</td>
<td>48</td>
<td>4</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>820</td>
<td>45</td>
<td>52</td>
<td>3</td>
<td>22</td>
<td>57</td>
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<tr>
<td>8</td>
<td>160</td>
<td>91</td>
<td>9</td>
<td>0</td>
<td>49</td>
<td>80</td>
</tr>
<tr>
<td>9</td>
<td>560</td>
<td>79</td>
<td>20</td>
<td>1</td>
<td>110</td>
<td>71</td>
</tr>
<tr>
<td>10</td>
<td>380</td>
<td>48</td>
<td>41</td>
<td>1</td>
<td>79</td>
<td>80</td>
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<tr>
<td>11</td>
<td>140</td>
<td>95</td>
<td>5</td>
<td>0</td>
<td>54</td>
<td>95</td>
</tr>
</tbody>
</table>

* BAL studies: per milliliter, total cell count per milliliter. AM, L, and N indicate relative proportion of alveolar macrophages, lymphocytes, and neutrophils in BAL. Lung function tests: CLdyn and TLCO were expressed as a percentage of predicted values for height-matched children, as indicated in "Materials and Methods" section.
cleotide probe was performed. Briefly, the PCR products were transferred onto a nylon membrane (Duralon-UV membranes; Stratagene), which was then prehybridized at 42°C for 2 h in 0.9 M NaCl/50 mM sodium phosphate/5 mM EDTA/1 mg/mL each of BSA, polyvinylpyrolidone, and Ficoll/50% formamide/0.3% SDS/0.25 mg/mL salmon sperm DNA. The filters were then hybridized overnight at 42°C to the 32P-labeled probe that was 5' end-labeled with (γ-32P) ATP (New England Nuclear; Boston) using T4 polynucleotide kinase and purified by centrifugation through small columns of Sephadex G-50 (Bio-Rad; Hercules, Calif). After washing at 50°C in 1×SSC/0.1% SDS two times for 20 min, the membrane was applied to X-ray film to obtain an autoradiographic image. As shown in Figure 1, hybridization of the membranes of the PCR products with labeled probes showed bands on autoradiographs corresponding in size to the ethidium bromide-stained bands.

Statistical Analysis of Data

Results were expressed as mean±SEM. The Kolmogorov-of-fit test was performed to test the normal distribution of the data in the control group. A Mann-Whitney U test was used to assess age-related differences between the group of control children who were younger than 2 years old and the group of older children. A Wilcoxon matched-pairs test was used to compare cell differentials and lung function parameters before and after follow-up. A Mann-Whitney U test was used to compare BAL and pulmonary function parameters in presence or absence of cytokine expression. A stepwise multiple regression analysis was performed to study the correlation between changes in the number of expressed cytokines and changes in BAL and pulmonary function parameters between the two studies. A p level <0.05 was considered statistically significant.

RESULTS

Differential Cytology in BAL Samples

Control Group: BAL was well tolerated by all the children, without complications. As indicated in the "Materials and Methods" section, no evidence of lung parenchyma abnormalities could be observed based on complete pulmonary investigations.

For each patient, the percent of fluid recovered (volume returned/volume infused×100), the number of cells/mL in the pooled sample, and the percent of alveolar macrophages, lymphocytes, and neutrophils

<table>
<thead>
<tr>
<th>mRNA</th>
<th>5' primers</th>
<th>3' primers</th>
<th>PCR Product Size, bp*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>5'-CATGCCATCTCGCTGGAC-3'</td>
<td>5'-CCACATCTGCTGGAGTG-3'</td>
<td>546</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-GCGAGAAGCTTACGGTCCG-3'</td>
<td>5'-CAGCAGCAGGTACAGAT-3'</td>
<td>603</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-GCACTGAGCATGTCGCCG-3'</td>
<td>5'-AGAGAGGAGGTTGACTGG-3'</td>
<td>510</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-ATGAGCCGCCCCACACAGA-3'</td>
<td>5'-CATCCATTTTCTAGGG-3'</td>
<td>190</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5'-GCCCTGGACACCAACTATG-3'</td>
<td>5'-GCTGACCTGGAGGACG-3'</td>
<td>336</td>
</tr>
</tbody>
</table>

*bp=base pair.

Figure 1. Cytokine expression profiles by alveolar cells obtained from patient 5 during the first study (A) and the second study (B). For actin and each cytokine are shown the photograph of ethidium bromide-stained agarose gel (columns 1), and the autoradiography of hybridization with the end-labeled cDNA specific probe (columns 2). M indicates the base-pair ladder.
are indicated in Table 1. No eosinophills were observed. The number of cells/mL return showed large variation with a range from 90 to 650×10³ cells/mL. Most cells collected were macrophages, with the following mean values (±SEM). Expressed as cells/mL return the number of macrophages was 316.8±10³ cells (±44.13×10³ cells) with a range of 79 to 666×10³ cells, and their relative proportion was 89.9% (±1.3%). Lymphocytes were the next most frequent cell type. The lymphocytes/mL return ranged from 3 to 67×10³ cells with a mean of 24.87×10³ cells (±4.78×10³ cells); their relative proportion was 8.9% (±1.4%). Neutrophils were present in ten children. Neutrophils/mL return ranged from 0 to 7×10³ cells with a mean of 2.27×10³ cells (±0.63×10³ cells); their relative proportion was 1.2% (±0.3%). The patterns of data distributions were evaluated by visual inspection of the observed histograms with the superimposed line representing the normal distribution and were analyzed by the Kolmogorov tests. In the study population, results of cells/mL return and of percentages of cells for each cell type gave histograms that appeared to follow a normal distribution (figures not shown). This was confirmed statistically: for each set of data, the distribution was not significantly different from the normal distribution.

Results from children younger than 2 years of age and from older children were then compared. No significant difference could be observed for the relative proportions of macrophages, lymphocytes, and neutrophils. The only parameter that appeared to be affected by age was the recovery of the fluid: the percentage of fluid recovered was higher in the younger population (p=0.04).

Group With Sarcoidosis: A significant increase in the proportion of BAL lymphocytes was observed in patients with sarcoidosis in the two studies performed (mean±SEM): 29.4±5.5%, and 26.8±4.5%, respectively (individual values are listed in Table 2). This increase was associated with a parallel decrease in the percentage of macrophages. Comparison between the first study and the second study did not show significant differences in the relative proportions of cells.

### Cytokine mRNA Expression

Control Group: No expression of IL-1β, TNF-α, IL-6, or TGF-β messenger RNA (mRNA) could be detected (data not shown).

Group With Sarcoidosis: Results are presented in Table 4. During the first study (Table 4, left), transcripts for IL-1β were detected in each case; TNF-α mRNA was observed in BAL cells of eight patients, IL-6 mRNA in BAL cells of five children, and TGF-β mRNA in four cases. It is interesting to note that TGF-β was found in those patients who also displayed expression of TNF-α and IL-6 mRNA, and that, in cases showing presence of IL-6 mRNA, TNF-α expression was always associated.

During the second study performed 6 months later, modifications in the cytokine profile were observed, as indicated in Table 4, right. Again, TGF-β transcripts were always found to be associated with the presence of TNF-α and IL-6 transcripts. IL-6 mRNA was detected in the BAL cell preparation of those patients who also displayed expression of TNF-α gene.

### Cytokine mRNA Expression and BAL Cytologic Studies

In the group with sarcoidosis, results of the cytokine studies showed permanent expression of IL-1β and changes in the expression of the other cytokines. To determine whether a relationship between expression of TNF-α, IL-6, or TGF-β, and abnormalities in BAL cellular profiles could be documented, statistical analysis was performed on pooled data of both studies comparing BAL parameters in presence or absence of mRNA expression for a given cytokine. No statistically significant difference could be observed between the

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**Table 4—Cytokine mRNA Expression in BAL Cells From Children With Sarcoidosis**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Actin</th>
<th>IL-1β</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>TGF-β</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
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<td>+</td>
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</table>

*Presence (+) or absence (−) of mRNA for actin, IL-1β, TNF-α, IL-6, and TGF-β in the first study (left) and the second study (right).
level of increase in lymphocyte population and the presence or absence of TNF-α transcripts. By contrast, expression of IL-6 mRNA or TGF-β mRNA was associated with an increased percentage of lymphocytes in the lavage fluid: p=0.03 and p=0.01, respectively.

The next statistical analysis was performed to determine whether changes in the profile of cytokine expression during the follow-up were associated with significant modifications in the cellular populations present in the alveolar space. For this analysis, results obtained during the first study and the second study were compared. For each individual, the difference in the number of expressed cytokines (TNF-α, IL-6, or TGF-β) between the first and the second study was assessed. A significant correlation was found between an increase in the number of cytokine mRNA expressed and an increase in the percentage of lymphocytes in the BAL (r=0.84, p<0.001).

Cytokine mRNA Expression and Pulmonary Function

In the group with sarcoidosis, results of pulmonary function tests (listed in Table 3) showed impairment of either CLdyn or TLCO in the first study with the following values (mean±SEM): CLdyn, 65.4±7.9%; and TLCO, 70.4±5.2%. In the second study, a decrease in CLdyn was still observed: 67.6±6.8%; the values of TLCO were within the normal range: 89.3±6.2%. Comparison between the first study and the second study did not show any significant differences in lung function parameters except for TLCO (p=0.04). To determine whether a relationship between expression of TNF-α, IL-6, or TGF-β, and abnormalities in pulmonary function could be documented, statistical analysis was performed on pooled data of both studies comparing results of lung function tests in presence or absence of a given cytokine expression. No statistically significant difference in CLdyn or TLCO values could be observed for any individual cytokine.

Using the same approach as indicated for BAL parameters, stepwise multiple regression analysis showed a significant association between an increase in the number of cytokine mRNA expressed during the follow-up and a decrease in CLdyn values (p=0.007). This correlation was independent of the variations of the BAL lymphocyte percentage (multiple r, including variations in lymphocyte percentage, was 0.94). By contrast, no statistically significant association could be observed between modifications of the number of cytokine expressed and changes in TLCO values.

DISCUSSION

Results presented in this report provide important information for the use and development of BAL as a clinical and research tool in the pediatric population. First, they define the differential cytology of BAL fluid in a control population and its distribution. Second, to our knowledge, they represent the first investigation of alveolar cell-derived cytokine expression in children without and with lung parenchyma disease.

Definition of normal BAL parameters is difficult in the pediatric population. As mentioned above, for ethical reasons, this definition can rely only on retrospective analysis of data from children who have undergone a complete pulmonary investigation. We were extremely careful in the selection of the control group whose recruitment was based on clinical, radiologic, microbiologic, and functional parameters and required 3 years. Two conclusions may be drawn from the results of the differential cytology presented herein. First, data from a control pediatric population appear to be similar to the results reported in several control nonsmoking adult populations. This observation, however, is not in concordance with the results recently reported by Ratjen et al. These authors have indicated a higher relative proportion of lymphocytes in their pediatric population with a mean of 16.2%, an SD of 12.4%, and a median of 12.5%. Moreover, 25% of their patients had a lymphocyte percentage that exceeded 20%. As they discussed in their work, their results may in fact be explained by the criteria used for patient selection. Indeed, their study population consisted of 48 children who had BAL under general anesthesia before elective surgery for nonpulmonary illnesses. The only information on the lung status of these patients was that they were without chronic respiratory symptoms and that none was receiving any medications at the time of investigation. One could therefore suggest the possibility of a mild form of alveolitis in some of the subjects despite the absence of symptoms.

Analysis of data distribution in our control group indicated that it was not statistically different from a normal distribution. This is likely to be explained by the selection criteria. This hypothesis is supported by the study performed in 42 asymptomatic adult patients by Laviolette. When this author excluded the five subjects who had lymphocyte fractions higher than 20%, lymphocyte percentages were normally distributed. The interpretation of the statistical study of the results presented by Laviolette raised again the difficult issue of patient selection, as discussed by Merchant et al.

The second conclusion drawn from the differential cytology data is that no significant differences could be observed between infants and older children, except for the percentage of fluid recovered. This is of interest considering the changes in the potential of alveolar multiplication observed before and after the mean age of 2 years, and possibly the changes in the local
involvement of growth factors which may exert different effects on alveolar cellular populations.29

Results presented herein also document expression of several cytokines by BAL cells from children without and with parenchymal lung disease. The protocol used in this study was chosen to provide information on the cytokine burden within the alveolar space of the patients and for this purpose was done on freshly isolated BAL cells without separation of the different cell populations. One interesting aspect of this protocol is that it eliminates phenotypic modifications of BAL cells that may be induced by cell manipulation and culture, as demonstrated by other studies.21 Another point of interest of our protocol is that it establishes simple BAL cell RT-PCR methods to evaluate inflammatory processes in the alveolar space, an important restraint on the development of BAL cellular studies in children with the limited quantity of lavage sample available. It is clear that this technique has limitations. It does not provide information on which cells express the cytokines. Also it does not indicate whether the corresponding protein is present within the alveolar space and active. However, as discussed by many authors, at the present time, no reliable methods of cytokine quantification in BAL fluid are available.13,22,23

In this study, we focused on IL-1, TNF-α, IL-6, and TGF-β expression. Our data indicated that IL-1β was expressed in all patients with sarcoidosis, but not in the group without pulmonary abnormalities. These results are interesting as they document the absence of IL-1β gene expression in a control pediatric population. Studies reported in adults indicate that controversies exist regarding the basal production of IL-1β in control subjects.27,23,24 These discrepancies have to be interpreted considering the experimental protocols used. As for IL-1β, TNF-α mRNA could not be detected in our group of children without pulmonary abnormalities, suggesting that this cytokine may not be expressed under control conditions in the pediatric population. In adults, conflicting data on production or expression of TNF-α by control alveolar macrophages (AMs) have been reported.15,21,23,24 In our group with sarcoidosis, in contrast with the results obtained for IL-1β, TNF-α transcripts were not found in all the children. Although no relationship could be documented between the presence of TNF-α transcripts in BAL cells and the level of alteration of pulmonary function test results, it may be suggested that expression of TNF-α was observed in those patients who displayed a more active form of the disease than those without TNF-α mRNA, the term active being used here with caution. Indeed, as discussed in a recent report, activity in sarcoidosis remains not precisely defined and therefore the tests needed to stage its degree are not clearly determined.25

In this study, we have used the functional and biological tests available at the present time in children. They are limited and they can detect only rough modifications in disease stability.

Our results on IL-6 expression indicated that this cytokine was mainly expressed in patients with alteration of the functional and biological parameters tested. The absence of IL-6 mRNA in children without pulmonary abnormalities is in agreement with the results of Becker et al.26 Classically, IL-6 is described as an important regulator of inflammation and immunity.27 IL-6 functions during the course of sarcoidosis remain unclear, but studies focusing on the effects of IL-6 on skin may provide some interesting insights. Turksen et al28 have observed that transgenic mice that overexpressed IL-6 in basal epidermal cells displayed increased skin protection from injuries or infection. Thus, in this regard, it could be suggested that in other organs such as the lung, IL-6 may play a central role in disease progression by counteracting the deleterious effects of local mediators in tissue remodeling. However, IL-6 may be viewed as a deleterious molecule, favoring sarcoidosis progression. In a recent article, Zhang et al29 have provided information suggesting that in active tuberculosis, the role of IL-6 may be predominantly negative leading to a dissemination of the disease.

Data reported herein indicated that expression of TGF-β mRNA by BAL cells was observed only in children with severe forms of pediatric sarcoidosis, based on functional and cytologic tests. In a recent study, Broekelmann et al30 have demonstrated that activated AMs expressed abundant TGF-β mRNA in idiopathic pulmonary fibrosis and they suggested that TGF-β synthesized by AMs and associated with extracellular matrix may serve as a chronic stimulus for persistent expression of connective tissue genes by resident fibroblasts and for development of fibrosis. Such mechanisms may be proposed in tissue remodeling processes associated with sarcoidosis.31

To our knowledge, the present study is the first report of cytokine expression profiles during the course of sarcoidosis in children. As shown herein, the examined cytokines were not expressed at the same time. Different profiles were individualized which seemed to be related to the activity and/or the severity of the disease, based on biological and functional parameters discussed above. IL-1β mRNA alone or in association with TNF-α mRNA appeared to be a pattern of cytokine expression present in the mild forms of sarcoidosis. In the more severe forms, in addition to the presence of transcripts for these two cytokines, TGF-β and IL-6 mRNAs were found. These data raise two comments. First, in several pediatric interstitial lung disorders, including sarcoidosis, a critical question for the physicians relates to the optimal management of
steroid therapy, knowing its toxicity especially in children. Study of BAL cytokine expression profiles may provide additional support for the decision of steroid treatment duration. Second, the different patterns of cytokine reported herein suggest a sequence in the involvement of these molecules during the course of the disease in children, which well fits in with the concept of cytokine networking. Indeed, they share similarities with the results of cellular studies performed using murine models of pulmonary granulomatous inflammation documenting sequential mononuclear phagocyte cytokine production of IL-1β, then TNF-α and IL-6. Several cellular and molecular studies give support to this sequential involvement of cytokines. IL-1β has been shown to induce NF-kB as well as AP-1 activity, and many IL-1β-induced genes, including TNF-α have NF-kB and AP-1 site in their 5′ flanking regions. Moreover, Tanner et al have shown that IL-1β increased TNF-α receptor expression. Induction of IL-6 and TGF-β in a later stage of the disease can be discussed using information obtained from several cellular studies. Elias and Lentz have documented the ability of combined IL-1β and TNF-α to stimulate IL-6 production. Also, Phan et al have demonstrated synergistic effects of IL-1β and TNF-α on induction of TGF-β production by endothelial cells.

In conclusion, results presented in this report document differential cytology and cytokine response of alveolar cells in children without and with interstitial lung disease. Long-term longitudinal studies of cytokine pattern dynamics are currently being pursued to give more support to the conclusions regarding the temporal sequence of cytokine gene expression in BAL cells during progression of lung inflammatory processes. It is likely that characterization of patterns of cytokine expression during the course of interstitial lung diseases in children would be of great interest for evaluation of disease severity and/or activity and therefore for planning of therapy.

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