INCREASED LEVELS OF INTERLEUKIN-12 AND INTERLEUKIN-18 IN BRONCHOALVEOLAR LAVAGE FLUID OF PATIENTS WITH PULMONARY SARCOIDOSIS

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We studied prospectively 43 patients with sarcoidosis and 13 normal subjects. IL-12 and IL-18 levels were measured using ELISA kits. Spirometry and body plethysmography were performed using an Elite DL Medgraphics body box. The sarcoidosis group was characterized by significantly higher median range of the plasma angiotensin-converting enzyme (ACE) concentration (72 vs. 34 U/l, P<0.0001), lymphocyte (34 vs. 14%, P<0.0001), CD4+ cells percentages (59 vs. 36%), and CD4/CD8 ratio (4.2 vs. 1.99, P<0.0001). The BALF IL-12 and IL-18 levels were significantly higher in sarcoidosis patients than in healthy subjects (4.1 pg/ml vs. 3.2 pg/ml; P<0.001 and 11.1 pg/ml vs. 6.15 pg/ml, P<0.0001, respectively). A negative correlation between BALF IL-12 and ACE plasma levels (r=-0.33, P<0.05) within the sarcoidosis group was found. Our data suggest a potential role of IL-12 and IL-18 in a local immunologic response in pulmonary sarcoidosis.

Key words: BALF, IL-12, IL-18, sarcoidosis, T cell subsets

INTRODUCTION

Sarcoidosis is a multisystem disorder of unknown etiology characterized by increased cellular immune responses at sites of disease activity, which is characterized by the formation of non-caseating granulomas in affected organs, most commonly in the lung (1, 2). The primary manifestation of the disease is an accumulation of mononuclear inflammatory cells, mostly activated CD4+ type 1
helper T cells, releasing interleukin IL-2 and interferon IFN-γ (3). More recently, the role of IL-12 and IL-18, reflecting T cell activity of the sarcoid immunologic response has been studied, indicating their involvement in (4, 5). The aim of the present study was to investigate the IL-12 and IL-18 concentration in bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis and to correlate this with disease activity markers.

MATERIAL AND METHODS

The study protocol was approved by the Ethics Committee of Bialystok Medical University in Bialystok, Poland. Each subject gave written informed consent for participation in the study.

Forty three non-smoking patients (23 women, 20 men) of median age 39.7 years (range 29–64 years) with sarcoidosis and 13 normal non-smoking subjects (6 women, 7 men) of median age 48 years (range 39–63) were studied. Diagnosis was based on medical history, clinical symptoms (cough and/or exertional dyspnea), standard chest radiography, CT scanning, lung function tests, and laboratory test results (serum angiotensin-converting enzyme - ACE) following the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Granulomatous Disorders statement (1). According to chest radiography staging of sarcoidosis, all 43 patients had stage II disease confirmed with high resolution computed tomography (HRCT) findings, which were lymphadenopathy and parenchymal opacities. The diagnosis was confirmed by lung transbronchial biopsy specimen. None of the patients received oral or inhaled glucocorticoids or antibiotics during the 6 months preceding the study. They were lifetime nonsmokers and did not experience any acute respiratory illness during the 6 weeks prior to the study.

Lung function and DLCO tests were performed with a body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to the American Thoracic Society/European Respiratory Society guidelines (6). Bronchoscopy and BALF processing was performed as a part of routine clinical management, according to the recommended guidelines and previous reports (7, 8). Subjects underwent bronchoscopy by flexible fiberoptic bronchoscope (Pentax FB 18 V; Pentax Corporation, Tokyo, Japan). Subjects received atropine (0.5 mg, i.m.), and midazolam (5 mg, i.m.). Local anesthesia was performed by inhalation of an aerosol solution of 22 ml of 2% lidocaine 15min before bronchoscopy. The bronchoscope was inserted and wedged in the right middle lobe, and three 50 ml aliquots of sterile saline solution, warmed to 37°C, were instilled into the subsegmental bronchus. Fluid was gently aspirated immediately after each aliquot was introduced, and collected in a sterile container. A second 50 ml aliquot of recovered fluid was labeled as BALF. BALF samples were analyzed for total and differential cell counts, flow cytometry to measure CD3+, CD4+, and CD8+ lymphocyte counts, and for IL-12 and IL-18 levels detected by ELISA.

During bronchoscopy, oxygen saturation and ECG tracings were continuously monitored. One aliquot was reserved for a total cell number using Nageotte’s chamber and these results were expressed as cells x 10^5/ml. The remaining fluid was immediately centrifuged at 800 rpm for 10 min at 4°C. The cell pellet was washed twice with phosphate-buffered saline solution (without Ca++ and Mg++). Cytocentrifugates were stained by the May–Grünwald–Giemsa method. The differential cell count of macrophages, neutrophils, lymphocytes, eosinophils, and epithelial cells was made under a light microscope (magnification x 1000) by counting at least 400 cells. Two observers blinded to the patients’ characteristics counted 400 cells to determine the differential cell count. A further 2 ml of BALF was resuspended in HBSS and layered onto a density gradient medium (Histopague-1077,
Sigma Diagnostics, UK) and cytocentrifuged at 600 x g for 10 min. The cell pellet was then resuspended in HBSS and incubated with 10% bovine serum albumin (Sigma Diagnostics, Poole, UK) for 20 min.

Flow cytometry was performed using an Epics XL flow cytometer (Coulter Electronics; Hialeah, Florida) that detects lymphocytes by fluorescence. To enhance the number of lymphocytes for the analysis, an acquisition gate was set using the lower third of the side scatter field. The analysis gate was broad in order to exclude non-lymphocytic cells. Results were expressed as a percentage of cells detected by fluorescence. Pairs of monoclonal antibodies to CD3+, CD4+, and CD8+ lymphocytes (Sigma Diagnostics) were then added to the suspension and incubated for 30 min, after which flow cytometry was performed.

IL-12 (Quantikine, R&D Systems, Minneapolis, MN) and IL-18 (MBL, Nagoya, Japan) were measured by commercially available ELISA kits, and all assays were performed according to each supplier’s recommendations. The minimum detectable doses of commercially available EIAs for IL-12, and IL-18 were typically less than 2.191, and 6.022 pg/ml, respectively.

Statistical analysis was performed using a statistical software package (Statistica for Windows, StatSoft, Tulsa, OK). The results are expressed as means ±SD or median (range), unless otherwise indicated. A Shapiro-Wilk W test for normality was applied to assess normality. A Mann Whitney U test for non-normally distributed variables was used. P<0.05 was considered to be statistically significant.

RESULTS

The demographic and clinical characteristics of subjects are reported in Table 1. The sarcoidosis patients had lower FEV1, RV, RV/TLC, and higher FVC, FEV1/TLC, SaO₂ with no significant difference in regard to TLC. The sarcoidosis group was characterized by a significantly higher median range of plasma ACE concentration (72 vs. 34 U/L, P<0.0001), with no difference in regard to the Ca²⁺ serum concentration and urine 24-h Ca²⁺ excretion compared with the control group (Table 1).

The number of cells in BALF was comparable in both groups (Table 1), the sarcoidosis patients had higher percentages of lymphocytes (34 vs. 14%, P<0.0001), CD4+ cells (59 vs. 36%) and a higher CD4/CD8 ratio (4.2 vs. 1.99, P<0.0001) (Fig. 1). The percentage of macrophages was significantly lower in the patient group (Table 1). All subjects tolerated bronchoscopy well, without any adverse events.

The BALF IL-12 levels were significantly higher in the sarcoidosis patients than in healthy subjects (4.1 pg/ml vs.3.2 pg/ml, P<0.001) (Fig. 2A). In addition, IL-18 levels were significantly increased in BALF samples (11.1 pg/ml vs. 6.15 pg/ml, P<0.0001) (Fig. 2B). In the sarcoidosis group, there was a negative correlation between the BALF IL-12 and ACE plasma levels (r=-0.33, P<0.05) (Fig. 3), with no correlations between IL-12 in BALF and other major disease activity markers (data not shown). There were no apparent correlations in regard to the BALF IL-18 levels (data not shown).
The present study demonstrates that the percentage of lymphocytes and the TCD4+, CD4/CD8 ratio in BALF from newly diagnosed, untreated patients with active pulmonary sarcoidosis were significantly higher than those in the control group. Despite several factors influencing the diagnostic accuracy of the differential cell count in BALF, the assessment of lymphocyte counts and CD4/CD8 ratios in BALF samples is still recommended in the clinical evaluation of patients with sarcoidosis (9-11). The number of lymphocytes in BALF in our patients is comparable with that obtained by others (12-15).

Assessing the IL-12 and IL-18 concentrations in BALF, we found the levels of both interleukins significantly higher in sarcoidosis patients compared with the control group. The level of the interleukins was studied by other authors in smaller groups of patients. Taha et al (16) found a significant increase in the number of cells expressing mRNA for IL-12R in active pulmonary sarcoidosis and active pulmonary tuberculosis, while allergic asthmatic patients exhibited a decrease in IL-12R mRNA-positive cells compared with normal control subjects. Colocalization studies demonstrate that IL-12R is expressed in both CD4+ and CD8+ cells of sarcoidosis patients. Shigehara et al (17) found that sarcoidosis patients are characterized by significantly elevated levels of IL-12 (p40 and p70) and IL-18 in BALF fluids compared with healthy subjects. IL-12 p70 and IL-18, immunohistochemically expressed in the epithelioid cells, were produced in sarcoid BALF cells and

### Table 1. Demographics, laboratory, and lung function tests in the sarcoidosis and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Sarcoidosis (n=43)</th>
<th>Control (n=13)</th>
<th>p</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>39.7</td>
<td>48.0</td>
<td>0.001</td>
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<tr>
<td>FEV1(%pred)</td>
<td>87</td>
<td>80</td>
<td>0.003</td>
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<tr>
<td>FVC (%pred)</td>
<td>96</td>
<td>85</td>
<td>0.066</td>
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<tr>
<td>FEV1/FVC (%pred)</td>
<td>99</td>
<td>85</td>
<td>0.003</td>
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<tr>
<td>TLC (%pred)</td>
<td>93</td>
<td>91</td>
<td>0.8</td>
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<tr>
<td>RV (%pred)</td>
<td>76</td>
<td>82</td>
<td>0.062</td>
</tr>
<tr>
<td>RV/TLC (%pred)</td>
<td>85</td>
<td>95</td>
<td>0.019</td>
</tr>
<tr>
<td>SaO2 (%pred)</td>
<td>97</td>
<td>91</td>
<td>0.20</td>
</tr>
<tr>
<td>Cytosis BAL</td>
<td>1000</td>
<td>1050</td>
<td>0.46</td>
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<tr>
<td>Macrophage BALF (%)</td>
<td>63</td>
<td>81</td>
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<tr>
<td>Lymfocytes BALF (%)</td>
<td>34</td>
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<td>0.0002</td>
</tr>
<tr>
<td>Neutrophils BALF (%)</td>
<td>1</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Eosynophiles BALF (%)</td>
<td>1</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>CD4 BALF (%)</td>
<td>59</td>
<td>36</td>
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<tr>
<td>CD8 BALF (%)</td>
<td>19</td>
<td>17</td>
<td>0.566</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>4.2</td>
<td>1.99</td>
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<tr>
<td>Ca²⁺ plasma mmol/l</td>
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<td>2.3</td>
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<tr>
<td>Ca²⁺ 24-h urine mmol/l</td>
<td>5.9</td>
<td>5.7</td>
<td>0.196</td>
</tr>
</tbody>
</table>

Values are medians. NS- non significant.
Fig. 1. T cell subpopulations in BALF of patients with sarcoidosis in comparison with the control group. A – Lymphocytes, B - T CD4+ cells, C - T CD8+ cells, and D - CD4/CD8 ratio. *Significantly different vs. the control group; P<0.005.

Fig. 2. IL-12 (Panel A) and IL-18 (Panel B) concentration in BALF of patients with sarcoidosis in comparison with the control group. The levels of both interleukins were significantly higher the sarcoidosis patients; *P<0.001.
synergistically induced IFN-g production. Hata et al (18), comparing the serum concentration of IL-12 p40 with other clinical markers of disease activity, found that the circulating IL-12 p40 was highly increased in sarcoidosis patients and was correlated with the number of involved organs and ACE levels, and that the mRNA expression of IL-12 p40 was increased in sarcoid lymph nodes. Antoniou et al (5) studying the levels of IL-12 and IL-18 in BALF found that both interleukins were elevated in sarcoidosis patients in comparison with the control subjects. This is consistent with our present findings. In the present study, elevated IL-12 correlated with the serum level of ACE, which is a major disease activity marker, with no such correlation regarding IL-18. In an accompanying paper (19), we assessed the levels of IL-12 and IL-18 in hypersensitivity pneumonitis – a granulomatous interstitial lung disease of known etiology. We found that the IL-12 and IL-18 levels in BALF of those patients are elevated. That provides evidence supporting the commitment of lymphocytes to the Th1-type cytokine profile in sarcoidosis and other granulomatous pulmonary diseases. Further large-scale studies are needed to define the precise role of the interleukins IL-12 and IL-18 in the immunopathogenesis of such disorders.

Conflicts of interest: The authors reported no conflicts of interest in relation to this article.

REFERENCES


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