The aim was to find out how the IL-12 and IL-18 levels in the bronchoalveolar lavage fluid (BALF) correspond to the inflammatory activity of the hypersensitivity pneumonitis (HP). We studied 12 patients with HP and 13 normal subjects. IL-12 and IL-18 levels were measured using ELISA kits. We found a significantly higher plasma angiotensin-converting enzyme (ACE) concentration (55 vs. 34 U/L, \( P=0.0016 \)), lymphocyte percentage (57 vs. 14\%, \( P<0.001 \)), CD8+ cells (32 vs. 17\%, \( P<0.001 \)) and a lower CD4/CD8 ratio (1.2 vs. 2.0, \( P<0.0001 \)). The IL-12 and IL-18 levels in BALF were significantly higher in HP patients than in healthy subjects (3.9 vs. 3.2 pg/ml, \( P=0.003 \) and 14.2 vs. 6.15 pg/ml, \( P<0.0001 \), respectively). We found a strong positive correlation between IL-12 and the percentage of lymphocytes \( (r=0.68, P=0.015) \) and a negative one between IL-12 and the percentage of macrophages in BALF \( (r=-0.64, P=0.024) \). We conclude that upregulation of the Th1 cell cytokine profile may play a significant role in the pathogenesis of HP.

**Key words:** hypersensitivity pneumonitis, IL-12, IL-18, BAL, T cell subsets

**INTRODUCTION**

Hypersensitivity pneumonitis (HP) is an immunologically mediated interstitial lung disease. It results from repeated inhalation of various causative antigens in susceptible individuals (1). Thermophilic actinomycetes are the major antigens that cause farmer’s lung disease (2). The abnormalities in the
bronchoalveolar lavage fluid (BALF) cell counts are almost always seen in patients with HP, according to the stage of the disease (3). 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, IL-10, IL-12, and interferon IFN-γ, possibly playing a central role in granuloma formation (4, 5). More recently, IL-18 and TNF production by macrophages derived from the BALF of HP patient has been reported (6). The aim of this prospective study was to find out how the BAL IL-12 and IL-18 levels correspond to the inflammatory activity of the disease.

MATERIAL AND METHODS

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

We studied 12 patients (3 women, 9 men) of median age 41.7 years (range 28–52 years) with HP (Farmer’s lung) and 13 normal subjects (6 women, 7 men) of median age 48 years (range 39–63 years). The patients fulfilled the following diagnostic criteria: 1) a history of exposure to organic antigens; 2) clinical signs and symptoms consistent with HP; 3) radiographic features and/or functional abnormalities characteristic of interstitial lung disease; 4) evidence of serum precipitins against one or more organic antigens; and 5) increased lymphocytes in BALF. All 12 patients presented with a subacute form of disease. On high-resolution computed tomography, all patients showed widespread and dominant ground-glass densities, with only minor reticulation, and no honeycombing. None of the patients received oral or inhaled glucocorticoids or antibiotics during the 6 months preceding the study. They were lifetime non-smokers and did not experience any acute respiratory illnesses 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 (7). Bronchoscopy and BAL processing was performed as part of routine clinical management, according to the recommended guidelines and previous reports (8, 9). Subjects underwent bronchoscopy with a 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 15 min before bronchoscopy. 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 aliquot of recovered fluid was labeled as BALF. The BAL fluid 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 the 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, p40 subunit and IL-18 were typically less than 2.0 and 6.0 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 of data distribution. 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. There was no significant difference in regard to FEV1, FVC, TLC, RV/TLC, and oxygen saturation. The median range of plasma angiotensin-converting enzyme (ACE) level was significantly higher in the HP group (55 vs. 34 U/l, P<0.0016) compared with controls, with an insignificantly higher urine 24-h Ca$^{2+}$ excretion in the HP patients (7.05 vs. 5.7 mmol/l). There was no difference in regard to the Ca$^{2+}$ serum concentration between the two groups (Table 1).

The number of cells in BALF was comparable in both groups (Table 1). The HP patients had higher percentages of lymphocytes (57 vs. 14%, P=0.0005) and CD8+ cells (31.5 vs. 17%, P=0.0004) and a lower CD4/CD8 ratio (1.2 vs. 1.99, P=0.0001 (Fig. 1). Percentage of macrophages was significantly lowered in the HP group (Table 1).

The levels of both IL-12 and IL-18 in BALF were significantly higher in the HP patients than in healthy subjects (3.94 vs. 3.2 pg/ml, P<0.003; Fig. 2A and 14.17 vs. 6.15 pg/ml, P<0.0001; Fig. 2B, respectively).

In the HP group, there was a strong positive correlation between the value of IL-12 in BALF, on the one side, and the percentage of lymphocytes (r=-0.68, P<0.015) (Fig. 3A) and a strong negative correlation between IL-12 and the percentage of macrophages in BALF (r=-0.64, P<0.024 (Fig. 3B). There was no such correlation in regard to the IL-18 BALF levels (data not shown).
The present study demonstrates that the percentage of lymphocytes and CD8+ T cells in BALF from newly diagnosed untreated HP patients was significantly higher and the CD4/CD8 ratio was lower compared with the control group. According to published data, CD4/CD8 ratio in BALF from HP patients may vary according to the clinical course of the disease, the type of the inhalation antigen, the extend (loading) of the exposure to the sensitizing antigen, the immune susceptibility of the patients, smoking habits, etc (10-12). A high percentage of CD8+ cells found in our series may account for a protective effect against pulmonary fibrosis (13). Stercova et al (14) suggest that patients with a normal value of CD4/CD8 ratio in BALF may run more severe course of disease. Moreover, the same authors suggest that increased lymphocyte count in BALF could be a predictor of the strength of inflammatory activity, especially in patients with lasting exposure to the causative agent (14). The role of IL-12, and more recently IL-18, in the course of HP has been extensively investigated. In the present study, we found that the levels of both interleukins were significantly elevated in BALF compared with control subjects. Other authors have reported similar findings (5, 13, 15-18). Schuyler et al (15) reported that following inhalation of Saccharopolyspora rectivirgula there was a remarkable BALF neutrophilia, followed by lymphocytosis, the appearance of MIP-1α and MCP-1.
Fig. 1. T cell populations in BALF in patients with hypersensitivity pneumonitis (HP) in comparison with the control group. Significantly higher percentages of lymphocytes (Panel A; \( P=0.0005 \)), TCD8+ cells (Panel C; \( P=0.0004 \)), and a lower CD4/CD8 ratio (Panel D; \( P=0.0001 \)) characterized the hypersensitivity pneumonitis (HP) group.

Fig. 2. IL-12 (A) and IL-18 (B) concentration in BALF in patients with hypersensitivity pneumonitis (HP) in comparison with the control group. *\( P<0.003 \) - Panel A and *\( P<0.0001 \) - Panel B.
Moreover, they observed a striking increase in BALF IL-1α, IL-6, IL-12, and TNF-α (15). Butler et al (16) suggest that altered IL-4 mRNA stability correlates with Th1 and Th2 bias and susceptibility to hypersensitivity pneumonitis. The authors also showed that regulation of mRNA stability may serve as an important mechanism underlying Th1/Th2 immune polarization. Chen et al (17) found that the production of IL-18 and TNF-α was increased in patients with HP independently of LPS. The concentration of the LPS-stimulated IL-12 production positively correlated with the percentage of lymphocytes and negatively correlated with that of macrophages. Ye et al (18), investigating an oxidant-antioxidant imbalance in the pathogenesis of idiopathic pulmonary fibrosis, found both IL-12 and IL18 elevated in BALF in sarcoidosis and HP. These results are consistent with our findings. A significantly higher level of IL-12 in the present study strongly correlated with the lymphocyte count, with a negative correlation with percentage of macrophages. In an accompanying paper we also present data on the levels of IL-12 and IL-18 in sarcoidosis, suggesting the role of both cytokines in the course of the diseases (19). Taken together, these observations suggest that upregulation of the Th-1-dependent signalization, involving IL-12 and IL-18, may play an important role in the pathogenesis of HP.

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

REFERENCES


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