Induced sputum and bronchoalveolar lavage from patients with hypersensitivity pneumonitis

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Summary
Background and Aim: Hypersensitivity pneumonitis (HP) is an immunologically induced inflammation of the lung parenchyma, though bronchial airways may be also involved. The aim of this study was to compare the cellular profiles of induced sputum (IS) in patients with newly diagnosed HP to that of healthy subjects, and to examine the relationship between inflammatory cells from IS and BAL.

Methods: Nine HP patients and 9 healthy volunteers were studied. IS was obtained by inhalation of hypertonic saline solution in all subjects. Bronchoscopy was performed on a different occasion in all patients and in five controls.

Results: IS was well tolerated and preferred to BAL by all subjects. Both IS and BAL from HP patients showed a significant increase in total cells (P<0.02 and P<0.001) and in lymphocytes (P<0.02 and P<0.001) and a significant decrease in macrophages (P<0.05 and P<0.001), when compared with normal subjects. In HP patients, total cells number in IS was higher than that in BAL (P<0.02). Moreover, the percentage of lymphocytes was significantly lower in IS than in BAL (P<0.001). No significant relationship was found between total cells or inflammatory cells from IS and the corresponding ones from BAL and wide limits of agreement were found between lymphocytes from IS and BAL.

Conclusions: This study demonstrated that both BAL and IS from newly diagnosed HP patients contained significantly more total cells and lymphocytes, when compared to healthy subjects. Moreover, differential cell counts in HP patients showed that IS and BAL reflected different compartments of inflammation. Thus, IS could represent a complementary, but not alternative tool to bronchoscopy both in research and in the clinical monitoring of HP patients.

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Introduction

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is an immunologically induced inflammation of the lung parenchyma resulting from repeated inhalations of a variety...
of causal agents, including organic dusts and chemical agents. HP may occur in several clinical forms and may lead to irreversible pulmonary damage, depending on several factors, including the amount and duration of exposure to the antigen, the nature of causal agents, and the host response. The disease is characterised by a diffuse and predominantly mononuclear cell inflammation of the small airways and pulmonary parenchyma and the main pathophysiologic process of patients with HP is a T lymphocytes alveolitis. The analysis of cells recovered in bronchoalveolar lavage (BAL) has been extensively used as diagnostic tool since it reflects the inflammatory cell population of the lung interstitium. In HP patients, BAL shows an increased number of lymphocytes and the analysis of T cell surface phenotypes revealed a strong predominance of CD8+ T lymphocytes. In HP patients, the immune inflammatory process is not compartmentalised within the alveolar walls, but it can also involve the bronchial airways. In these patients, the bronchial epithelium may be extensively damaged during the active phase of disease, the computed tomography can show a variety of bronchial wall abnormalities and a bronchial responsiveness to methacholine may be found. The disease is characterised by a diffuse and predominantly mononuclear cell inflammation of the small airways and pulmonary parenchyma and the main pathophysiologic process of patients with HP is a T lymphocytes alveolitis. The analysis of cells recovered in bronchoalveolar lavage (BAL) has been extensively used as diagnostic tool since it reflects the inflammatory cell population of the lung interstitium. In HP patients, BAL shows an increased number of lymphocytes and the analysis of T cell surface phenotypes revealed a strong predominance of CD8+ T lymphocytes. In HP patients, the immune inflammatory process is not compartmentalised within the alveolar walls, but it can also involve the bronchial airways. In these patients, the bronchial epithelium may be extensively damaged during the active phase of disease, the computed tomography can show a variety of bronchial wall abnormalities and a bronchial responsiveness to methacholine may be found.

During the past decade, induced sputum (IS) by hypertonic saline has been widely used to assess airway inflammation in asthma and chronic obstructive pulmonary disease, since it provides reliable results, that are comparable to other invasive technique, such as mucosal biopsy and BAL. In fact, in asthmatics, eosinophil count of IS was significantly related to that of BAL, moreover, the percentage of CD4+ lymphocytes in IS has been found to significantly correlate with that in BAL. Recently, IS has been proposed in the assessment of interstitial lung disease, since it provided reliable results in pneumoconiosis, sarcoidosis, non-granulomatous interstitial lung disease, pulmonary involvement in Crohn’s disease, and Fabry’s disease.

Until now, the potential use of IS in the assessment of pulmonary inflammation due to HP has been poorly examined. Additionally, in HP patients the cellular profile analysis of proximal airway samples, such as IS, could add information to that coming out from the assessment of more distal samples of airways, such as BAL. Therefore, the aim of this study was to compare the cellular profiles of IS in patients with newly diagnosed HP to that of healthy subjects, and to examine whether or not inflammatory cell counts from IS are related to those from BAL.

**Methods**

**Subjects**

We studied nine patients (two female, age range 30–70 years, all non-smokers) affected by HP. Diagnosis was based on symptoms (cough and/or exertional dyspnoea), standard chest X-ray, high-resolution computed tomography (HRCT) and laboratory test results (serum antibody to *Micropolyspora Faeni* in seven patients and serum antibody to avian droppings in two patients). According to HRCT patterns, eight patients had subacute HP (four patients had patch ground glass opacity, three had hazy small nodular opacities, and 1 had small amount of fibrosis) and one patient had chronic HP (fibrosis with traction bronchiectasis and variable amount of honeycombimg). None of the patients received oral or inhaled corticosteroids or antibiotics during the 4 months preceding the study.

The control group included nine healthy volunteers (six female, age range 21–40 years) recruited among medical students and hospital staff. They were lifetime non-smokers and did not experience any acute respiratory illness in the 4 weeks prior to the study. Each subject gave informed consent to the study protocol.

**Lung function test**

Pulmonary function was measured by a flow-sensing spirometer connected to a computer for data analysis (Vmax 22, Sensor Medics, Yorba Linda, US). Forced vital capacity (FVC), forced expiratory volume at the 1st second (FEV1), and forced mid-expiratory flow (FEF25−75) were recorded. Carbon monoxide transfer coefficient (KCO) was also measured by the single breath method. All these variables are expressed as percent of predicted value.

**Induced sputum and processing**

Sputum was induced by the inhalation of hypertonic saline as previously described. All subjects inhaled 3% hypertonic saline solution four times for 5 min using an ultrasonic nebulizer (Heyer Orion 1, BAD EMS; mean volume output: 2.40 ml/min). Throughout the procedure, subjects were encouraged to cough and to expectorate into a plastic container. Three flow volume curves were performed before and after each inhalation and the best FEV1 was recorded. Induction of sputum was stopped if FEV1 value fell by at least 15% from baseline or if troublesome symptoms occurred.
The volume of the sputum sample was measured and an equal volume of dithiotreitol 0.1% was added and incubated at 37°C for 30 min. Ten microlitres of the homogenised sample were used to determine the total cell count and results were expressed as number of cells \times 10^5/ml. The remaining sputum was washed with phosphate-buffered saline and centrifuged at 2000 rpm for a 5-min period. The supernatant was aspirated, and cell pellets were re-suspended in saline (2000 rpm for 10 min), cytocentrifuged at 600 rpm for 10 min and stained with May-Grnwald-Giemsa. The sample was considered adequate if the squamous cells percentage was less than 20%. The percentage of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were counted by two of the authors (R. D. and S. M.) on at least 400 cells, excluding squamous cells.

**Bronchoscopy and BAL processing**

At least 2 days after IS, subjects underwent bronchoscopy by flexible fibroptic bronchoscope (Olympus 1T10; Tokyo Japan). Subjects were treated with atropine (0.5 mg intramuscularly). Then local anaesthesia was performed by inhalation of an aerosol solution of 22 ml of 2% lidocaine followed by the sucking of a 20-mg tablet of tetracaine 15-min before bronchoscopy. BAL was carried out as previously described. The bronchoscope was wedged into a segment of the right middle lobe, and three 50-ml aliquots of sterile saline solution, warmed at 37°C, were instilled into the subsegmental bronchus. Fluid was gently aspirated immediately after each aliquot was introduced and collected in a sterile container. During the bronchoscopy, oxygen saturation and ECG tracing were continuously monitored.

An aliquot was reserved for total cell number using a Nageotte’s chamber and results were expressed as cells \times 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^{2+} and Mg^{2+}). Cytocentrifugates were stained by the May-Grnwald-Giemsa method. The differential cell count of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells was made under light microscope (magnification x 1000) by counting at least 400 cells. Two of the authors (R. D. and S. M.) independently performed differential cell counts without any knowledge of subjects characteristics. Bronchoscopy was performed in all patients with HP and in five out of nine normal subjects.

**Statistical analysis**

Data were tested for normal distribution by the Kolmogorov test and expressed as means \pm SD. Differences between cell counts in IS and BAL from patients with HP and from normal subjects were analysed by unpaired Student’s t-test. Differences between total and differential cell counts from IS and BAL in patients with HP were evaluated paired Student’s t-test. Correlations between different cells from different samples were examined by Pearson’s correlation coefficient. The agreement between IS and BAL from HP patients was assessed by the method of differences against the means according to Bland and Altman.25 Probability values of P<0.05 were accepted as significant.

**Results**

Characteristics of subjects are reported in Table 1. Patients with HP were significantly older (P<0.01) and had lower values of FVC (P<0.02), FEV\(_1\) (P<0.001), FEF\(_{25–75}\) (P<0.001), when compared to normal controls. Moreover, all patients had K\(_{CO}\) values less than 80% of predicted value (Table 1). All subjects well tolerated both IS and bronchoscopy procedure, without experiencing any adverse events. The sputum samples were adequate in all cases.

The results of total and differential cell counts in IS and BAL from patients with HP and normal subjects are reported in Table 2. With respect to IS samples, HP patients had a significant increase in total cells (81.0 \times 10^5/ml \pm 49 vs. 34.4 \times 10^5/ml \pm 21, P<0.02) and in lymphocytes (4.1 ± 3% vs. 0.8 ± 2%, P<0.02), and a significant decrease in macrophages (57 ± 31% vs. 81 ± 12%, P<0.05), when
compared with normal subjects (Fig. 1). Similarly, in BAL samples HP patients had a significant increase in total cells (38.5 ± 9.9 vs. 14.4 ± 36, $P < 0.001$) and in lymphocytes (57 ± 25% vs. 3 ± 3%, $P < 0.001$), and a significant decrease in macrophages (35 ± 21% vs. 95 ± 3%, $P < 0.001$), when compared to those of normal subjects (Table 2).

In HP patients, the number of total cells recovered in IS was higher than that in BAL samples ($P < 0.02$). Moreover, the percentage of lymphocytes was significantly lower in IS than in BAL ($P < 0.001$) (Fig. 2). The percentages of eosinophils and neutrophils were numerically, but not statistically, increased in HP patients, when compared to those of controls (Table 2).

No significant relationship was found between total cells or percentage of any other inflammatory cell from IS and the corresponding ones from BAL. Moreover, wide limits of agreement were found

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### Table 2  Cell counts from samples of induced sputum and BAL in HP patients and normal subjects.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IS HP patients ($n = 9$)</th>
<th>IS Normal subjects ($n = 9$)</th>
<th>BAL HP patients ($n = 9$)</th>
<th>BAL Normal subjects ($n = 5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells (no. $\times 10^5$/ml)</td>
<td>81 ± 49*#</td>
<td>34.4 ± 21</td>
<td>38.5 ± 9.9**</td>
<td>14.4 ± 36</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>57 ± 31*</td>
<td>81 ± 12</td>
<td>35 ± 21**</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>32 ± 35</td>
<td>15 ± 11</td>
<td>5 ± 8</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>4 ± 3*†</td>
<td>0.8 ± 2</td>
<td>57 ± 25**</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>4 ± 8</td>
<td>0.2 ± 0.2</td>
<td>3 ± 5</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>3 ± 4</td>
<td>4 ± 2</td>
<td>0.8 ± 0.5</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

* $P < 0.05$ vs. IS of normal subjects; *# $P < 0.05$ vs. BAL of HP patients; † $P < 0.001$ vs. BAL of HP patients; ** $P < 0.01$ vs. BAL of normal subjects.
between lymphocytes from IS and BAL. The bias between lymphocytes in IS and lymphocytes in BAL was \(-53\%\) and the limits of agreement ranged from \(-5\%\) to \(-101\%\) (Fig. 3).

**Discussion**

The results of this study demonstrated that not only BAL samples but also IS samples from newly diagnosed untreated patients with HP contained significantly more total cells and lymphocytes, when compared to those recovered in healthy subjects. As expected, lymphocyte counts in BAL samples from HP patients were higher than those in IS samples. Moreover, differential cell counts in the two different samples from HP patients showed a lack of correlation and thus suggest that IS and BAL reflected different compartments of inflammation. Finally, IS was well tolerated and preferred to BAL by all subjects.

Several factors are known to remarkably influence the diagnostic accuracy of differential cell count from BAL samples. Nevertheless, the assessment of lymphocytes count and CD4/CD8 ratio in BAL samples is still in use and recommended in the clinical evaluation of HP patients. The percentage of lymphocytes in BAL from our patients with newly diagnosed HP is comparable to that previously reported in several studies, ranging between 1.7% and 82%.

We found that the number of lymphocytes from IS in patients with HP was increased, as compared to that from healthy subjects. An increased number of lymphocytes in IS is of interest, since sputum samples are much more concentrated in cells than BAL. Surprisingly, the potential use of IS samples in these patients has received very little attention. As far as we know, IS as investigative tool in HP patients has been used only by Mroz and co-workers. In their recent report, the authors found a higher lymphocyte count in IS from HP and sarcoidosis patients, as compared to that from COPD patients. A control group of healthy subjects was not considered. Moreover, they demonstrated that the number of lymphocytes in IS from patients with HP is sufficiently high to identify CD4+ and CD8+ lymphocytes and assess the expression of their surface markers of activation. Interestingly, recent studies have demonstrated that indeed, this was also possible in asthmatics who showed rather low percentages of sputum lymphocytes, as well as in patients with sarcoidosis and with COPD. Whether this non-invasive and better tolerated procedure could be also useful in the assessment of disease activity in HP, especially in advanced forms and in the follow-up, is probably worth studying.

It is of interest that an increased percentage of lymphocytes was present in IS from most untreated patients with HP, as compared to that of healthy controls. However, unlike BAL, IS lymphocyte percentage could not discriminate clearly between HP patients and healthy controls, because of a broad overlap. Indeed, IS primarily samples the more proximal airways, whereas BAL mainly samples the alveolar component. Although the dynamics of lymphocytes in the lung has not yet been fully established, they could accumulate by active recruitment and selection, by local accumulation or as the consequence of decreased apoptosis. It is noteworthy that in patients with HP, lymphocytes from BAL showed an increased motility and an upregulation of alpha E beta 7 integrin expression that contributes to their selective accumulation. They may undergo an oligoclonal expansion and show a prolonged survival due to decreased apoptosis. The effect of microenvironment on sputum lymphocyte in HP patients is not known, but it is tempting to hypothesise that the bronchial as well as the alveolar compartment could contribute to their increased count. Interestingly, lymphocytes are the most frequent cells in bronchial biopsy from asthmatic patients, but very few are recovered in IS or BAL.

In conclusion, we demonstrated that cell counts in IS from patients with newly diagnosed HP do not reflect the inflammatory process of the alveolar compartment. However, the cell concentration and the percentage of recovered lymphocytes in sputum are higher than in healthy controls, though with considerable overlap. Thus, we would suggest that this inexpensive, non-invasive, and easily
repeatable method could represent a complementary, but not alternative tool to bronchoscopy both in research and in the clinical monitoring of patients with hypersensitivity pneumonitis.

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References

31. Leckie MJ, Jenkins GR, Khan J, et al. Sputum T lymphocytes in asthma, COPD and healthy subjects have the phenotype of