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Induced sputum as a tool for early detection of airway inflammation in connective diseases-related lung involvement

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Summary
Background: Induced sputum (IS) sampling is a safe and validated approach to study bronchial inflammation in chronic obstructive lung diseases. Although promising results have also been reported in various diffuse interstitial lung disorders, the potential use of IS in the assessment of connective tissue diseases (CTD)-related lung involvement has not yet been investigated.
Aim of the study: To evaluate the clinical usefulness of IS in the early management of patients suffering from rheumatoid arthritis (RA) and systemic sclerosis (SSc) at the onset of respiratory symptoms.
Patients and methods: The study population included 19 patients (RA = 12; SSc = 7) and 14 age- and sex-matched healthy volunteers. Lung function testing, high resolution computed tomography (HRCT) of the thorax and IS collection were performed in all cases. Broncho-alveolar lavage (BAL) was obtained in selected patients.
Results: IS samples from patients contained a significantly higher percentage of neutrophils and a lower percentage of macrophages compared to healthy subjects ($p = 0.002$ and 0.001, respectively), while the total cell number showed no differences. In addition, sputa yielded both higher cell counts and higher neutrophils than BAL samples ($p = 0.02$ in all instances). No correlations were found between IS findings and lung function parameters, HRCT and BAL findings.

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Conclusions: This is the first study investigating the inflammatory cell pattern in IS from CTD patients with early clinical evidence of lung involvement. Future studies are needed to determine whether the assessment of airway inflammation adds significant information that may result in a relevant improvement of disease management.

Introduction

Lung involvement is a quite frequent feature of connective tissue diseases (CTD). Diffuse interstitial lung disease (ILD) is the most common pulmonary manifestation in patients affected by rheumatoid arthritis (RA), being a progressive disease often leading to severe symptoms and respiratory failure.[1] Prevalence of ILD in RA varies according to the diagnostic criteria used, ranging from low rates, based on conventional chest X-ray, to up to 71%, based on the evidence of alveolitis.[2] Pulmonary fibrosis is even more common in systemic sclerosis (scleroderma-SSc) as it plays an important role in the disease prognosis and outcome, suggesting that early detection of lung abnormalities is of high priority.[3]

Several possibilities are available for the assessment of interstitial lung involvement in the course of CTD. Unlike chest radiography which may be normal in symptomatic patients, thoracic high resolution computed tomography (HRCT) is more sensitive and accurate in detecting early changes of lung fibrosis.[4,5] In a similar manner, unlike decreased forced vital capacity (FVC) and total lung capacity (TLC), the single breath diffusion capacity of the carbon monoxide ($DLCO_{sb}$) has been demonstrated to be a highly sensitive method and an appropriate tool for the detection of early lung involvement.[6] Evaluation of diffusion capacity may however not provide enough information on the extent of lung inflammation and fibrosis. In this issue, broncho-alveolar lavage (BAL) collection is regarded as a safe and inexpensive procedure for the investigation of various forms of ILDs and their activity.[5] In SSc, even single-site BAL usually correctly detects the presence of alveolitis.[7] BAL is often abnormal in asymptomatic patients with early alveolitis who have normal chest radiographs, providing information about the nature of the ongoing subclinical disease that may be useful for the estimation of prognosis and choosing the appropriate treatment.[5,8]

Sputum induction by hypertonic solution is a validated method to study bronchial inflammation in chronic obstructive pulmonary diseases (COPD), including asthma and chronic bronchitis.[9–11] Unlike mucosal biopsies and BAL, whose sampling requires more invasive procedures, collection of induced sputum (IS) has been shown to be safe and well tolerated irrespective of the method used.[12] IS has already provided promising results in patients with ILDs,[13,14] including pneumoconiosis,[15,16] sarcoidosis,[17,18] hypersensitivity pneumonitis,[19] non granulomatous ILDs,[20] and lung involvement in Crohn's and Fabry's diseases.[21,22] However, to our knowledge, the potential use of IS in the assessment of pulmonary inflammation in CTD has been poorly investigated until now.[23]

Aim of the present study was to evaluate the cellular pattern and clinical usefulness of IS in the early management of patients suffering from CTD, including RA and SSc, at the onset of respiratory symptoms. Moreover, we asked whether IS findings were correlated with lung function, X-ray and BAL data.

Methods

Study population

The study population included 19 patients meeting the criteria for the diagnosis of RA ($n = 12$) and SSc ($n = 7$).[24,25] All patients were enrolled at the Division of Rheumatology of the University "Federico II" of Naples and referred to the Division of Respiratory Diseases of the same University at the Monaldi hospital, Naples, Italy. The control group included 14 healthy volunteers recruited among hospital staff. The study was approved by the local Ethics Committee and all subjects gave written consent.

Pulmonary function tests

Spirometry and haemoglobin (Hb)-adjusted-$DLCO_{sb}$ were performed using a computer-assisted spirometer (Master Screen Diffusion, Jaeger, Wuerzburg, Germany) according to the American Thoracic Society/European Respiratory Society standards.[26–28] Arterial blood gases were analysed at rest while subjects were breathing ambient air (FiO_2 21%).

Radiology

HRCT was realized with 1 mm thick sections at 10 mm intervals with prone slices to exclude dependent changes. CT scans were analysed according to the method of Remy Jardin and given a semi-quantitative score for the presence or absence of changes compatible with fibrosis or ground glass attenuation (0 = absent; 1 = mild; 2 = moderate; 3 = severe).[29]

Induced sputum collection and processing

Sputum induction was performed using a modification of the method described by Fahy et al.[30] Briefly, following premedication with 200 μg salbutamol, all subjects inhaled 3% hypertonic saline solution generated by a DeVilbiss 65 ultrasonic nebulizer (DeVilbiss Corporation, Somerset, PA, USA) with a mean volume output of 2.4 mL/min. Throughout the procedure, subjects were encouraged to cough and to expectorate into a sterile plastic container. Ten minutes after the start of nebulization and every 5 min thereafter, they were asked to rinse their mouths with normal saline to

minimize contamination with saliva. Flow-volume curve monitoring was performed before and after each inhalation. Nebulization was stopped after 20 min or earlier if the FEV_1 value fell by at least 15% from baseline or if troublesome symptoms occurred.

Sputum samples (total volume of at least 2 mL) were processed within 2 h after collection. An equal volume of freshly prepared 0.1% dithiothreitol (DTT—Sigma Chemical Co., St Louis, MO, USA) was added and samples were incubated at 37 °C for 30 min. Ten microliters of each sample were used to determine the total cell count expressed as number of cells $\times 10^6$/mL. The remaining sputum was then washed with 5% foetal calf serum (FCS)-enriched phosphate-buffered saline (PBS) solution and centrifuged at 1200 rpm for 10 min. The supernatant was removed while cell pellets were washed twice with PBS (without Ca^{2+} and Mg^{2+}). Cytocentrifugates were stained by the May–Grunwald–Giemsa method. The differential cell count of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells was made under a light microscope (magnification $\times$ 1000) by counting at least 400 cells. A cut-off of 20% squamous cells was used for defining adequate samples.[11] All analysed sputa were suitable, as patients not able to expectorate adequate sputum samples were initially excluded from the study population.

BAL collection and processing

BAL collection was performed as previously described.[31] Briefly, three 50 mL aliquots of 0.9% saline solution were instilled through a flexible bronchoscope (Olympus BF 240, Tokio, Japan) in a segmental or sub-segmental bronchus of the involved lung lobe following local xylocaine anaesthesia. Samples were filtered through a sterile two-layer cotton gauze to remove mucus and centrifuged at 1000 rpm for 10 min at 4 °C. Recovered cells were resuspended in RPMI 1640 medium and counted. Cell viability (>90%) was determined by trypan blue exclusion assay. Staining with May–Grunwald–Giemsa was performed to evaluate the leukocyte differential count.

Flow cytometric analysis of T cell subsets

One aliquot of processed sputum or BAL samples was reserved for T cell subsets evaluation. Flow cytometry was performed on a FACScan equipped with an Argon laser (Becton-Dickinson, Mountain View, CA, USA). Data were analysed using the Cell Lysis II program (Becton-Dickinson). The selection of the lymphocyte population was based on side scatter and expression of CD45. Lymphocytic T subsets were identified by monoclonal antibodies (Mo Abs) as follows: CD3 for total lymphocytes; CD4 and CD8 for T cells. Mo Abs were directly conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Cells were incubated for 30 min and read either immediately or after 24 h. Matched isotype Abs were used as negative controls. All Abs used were purchased from Becton Dickinson.

Statistical analysis

Descriptive statistics were used to analyse continuous variables. Differences between lung function parameters from patients and controls were performed with the Student's *t*-test. Differences between cell counts and differentials in IS and BAL samples from patients and healthy controls were analysed with the Mann–Whitney *U*-test. The Wilcoxon test was used to compare differences in differential counts and cell subsets in IS and BAL from patients. Correlations between different cell subsets in different samples from patients were examined by Pearson's correlation coefficient. Spearman's rank correlation was used to evaluate any relationship between cell subsets, lung function parameters and thoracic HRCT findings. A p value <0.05 was considered significant. All analyses were performed with the SPSS.11 software.

Results

Demographics

Demographic characteristics of the study population are reported in Table 1. As no significant differences were found by comparing patients with AR with those affected by SSc, all of them were considered as a unique group. Patients with no previous history of lung diseases were included in the study early at the onset of pulmonary symptoms, which mainly included non-productive cough (47%) and/or dyspnoea upon exertion (63%). Respiratory infections, at least in the previous 2 months, were ruled out in all cases. At enrolment, all patients were under treatment with oral non-steroid anti-inflammatory drugs (diclofenac: $n = 2$; indomethacin: $n = 1$) or corticosteroids (prednisone: $n = 16$) in association, in same instances, with other immunosuppressive drugs (methotrexate: $n = 4$; cyclophosphamide: $n = 6$; cloroquine: $n = 5$).

Table 1 Demographics of the study population.

Healthy controls	14
Gender, F:M	8:6
Age, years†	41 ± 9.5 (37; 35–46.7)
Smoking status*	2 (14)
Patients	19
Gender, F:M	12:7
Age, years†	48.7 ± 13.3 (54; 39.7–60)
Smoking status*	3 (16)
AR*	12 (63)
SSc*	7 (37)
Disease duration, years†	3.4 ± 2.1
Anti-rheumatic therapy*,‡	19

*Data are expressed as *n* (%).
†Data are expressed as mean value ± SD (median value; IRQ 25–75).
‡Treatment regimens included non steroid-anti-inflammatory drugs, corticosteroids, methotrexate, cyclophosphamide, and chloroquine.

Lung physiology

Results of lung function testing are shown in Table 2. A mild to moderate restrictive ventilatory pattern was detected in patients as compared to controls due to the reduction of FEV_1, FVC, and TLC values (% predicted) ($p<0.0001$ and 0.002, respectively), while no differences were found by comparing the Tiffenaux index. To date, airflow obstruction ($FEV_1/FVC<70\%$) was evident in only 3 cases (16%). Interestingly, more than half of patients showed a significant reduction of FEF_{25-75} values ($p<0.0001$). Not surprisingly, the Hb-adjusted-$DLCO_{sb}$ values were significantly lower in patients than in control subjects ($p=0.002$), being <50% of predicted in four cases (21%).

Radiology

Lung HRCT was performed as part of a routine clinical protocol. Abnormalities compatible with interstitial lung disease, that are ground glass attenuation and thickened septal lines (scores = 1 and 2), were detected in more than half patients (respectively in 32% and 26% of cases). No evidence of end-stage lung fibrosis was found (score = 3) in any case. No abnormalities were found in 42% patients (score = 0). Additional findings included pleural effusion in 2 AR patients.

Induced sputum

Total and differential (%) cell counts in IS from patients and healthy controls are shown in Table 3. All subjects tolerated well sputum induction without experiencing adverse events. The number of total cells ($\times 10^6$/ml) was not different in patients compared to controls. However, both the percentage and the absolute number of neutrophils were significantly higher in patients ($p=0.002$ and 0.02, respectively), while the percentage of macrophages was lower as compared to controls ($p=0.001$). No other significant differences were found between the two study groups for any cell type. No correlation was found between the neutrophil differential percentages in IS and any lung function parameter or thoracic HRCT finding ($n=19$).

Broncho-alveolar lavage

In order to investigate features of pulmonary inflammation to be compared with IS findings, patients with HRCT evidence of lung involvement were asked to be submitted to FBS for BAL collection. Two patients out of 11 refused the exam. Data are reported in Table 4. The comparison of the total cell number recovered in IS and BAL samples from the 9 patients studied demonstrated that sputa yielded higher cell counts ($p<0.02$). When looking at cell differentials, a neutrophil alveolitis (neutrophils >10%) was present in 8 patients (88%), being more than 20% in two cases (27% and

Table 2 Lung function testing.*

Parameter	Controls ($n=14$)	Patients ($n=19$)	$p^\dagger$
PaO_2, mmHg at rest (21% FiO_2)	nd‡	90±5 (89; 86–93)	—
FEV_1, % predicted	104±8 (105; 100–112)	77±20 (82; 59–93)	<0.0001
FVC, % predicted	103±7 (100; 98–105)	83±15 (84; 75–93)	<0.0001
FEV_1/FVC, %	84±2 (84; 83–85)	79±12 (82; 71–89)	ns§
FEF_{25-75}, % predicted	112±13 (110; 103–124)	48±24 (43; 29–74)	<0.0001
TLC, % predicted	101±5 (100; 97–102)	88±13 (93; 81–96)	0.002
$DLCO_{sb}$, % predicted	103±6 (103; 97–106)	59±18 (55; 45–70)	<0.0001

*Data are expressed as mean value±SD (median value; IRQ 25–75).
†Unpaired Student's *t*-test.
‡Not determined.
§Not significant.

Table 3 Induced sputum differential cell count.*

	Cells $\times 10^6$/ml	% macrophages	% lymphocytes	CD4/CD8	% neutrophils	% eosinophils
Controls ($n=14$)	6±2.9 (4.2; 3.5–7.5)	60.6±18.6 (67.5; 52–71.5)	2.2±1.2 (2; 1.2–3)	1.8±0.9 (1.6; 1.2–2.1)	36.3±18.5 (30; 25.5–46.2)	0.8±1.3 (0; 0–1)
Patients ($n=19$)	7.2±4.7 (5.5; 3.2–9.9)	35±11 (30; 25–42)	3.5±1.9 (3; 2–4)	2.7±2.2 (1.0; 0.6–3.6)	59±11 (63; 54–68)	1.1±2.4 (0; 0–2)
$p^\dagger$	ns‡	0.001	0.042	ns	0.002	ns

*Data are expressed as mean value±SD (median value; IRQ 25–75).
†Mann-Whitney *U*-test.
‡ns = not significant.

Table 4 Comparison of broncho-alveolar lavage and sputum differential cell count*.

	Total cell count	% macrophages	% lymphocytes	CD4/CD8	% neutrophils	% eosinophils
Patients ($n = 9$)						
BAL ($\times 10^5$/mL)	3.5±0.7	63±19	11±6.1	1.3±1	16±11	8.7±10
	(3.7; 3–4.1)	(69; 62–73)	(15; 6–16)	(0.9; 0.7–2.1)	(12; 11–21)	(4; 2–11)
Sputum ($\times 10^6$/mL)	7.6±4.9	32±11	4±2.4	1.8±1.7	61±10	2±3.6
	(6.9; 3.2–10)	(30; 25–34.5)	(4; 2.5–4)	(1.3; 0.4–2.6)	(63; 59–64)	(0; 0–2)
$p^{\dagger}$	<0.02	0.02	0.02	ns‡	0.02	0.02

*Data are expressed as mean value±SD (median value; IRQ 25–75).
†vs sputum samples, Wilcoxon test.
‡ns = not significant.

41%, respectively). As expected, neutrophils were however more represented in sputa ($p = 0.02$), while macrophages, lymphocytes and eosinophils were higher in BAL samples ($p = 0.02$ in all instances). No difference of the CD4/CD8T cell ratio was found in BAL in comparison to induced sputa. No correlation was found between cell differentials in IS and BAL samples ($n = 9$).

Discussion

In the present study, the assessment of airway involvement in the course of CTD was evaluated by means of IS. Patients, including cases affected by AR and SSc with no previous history of lung diseases, were early recruited at the onset of pulmonary symptoms. The most striking observation was that IS samples from patients contained a significantly higher percentage of neutrophils and a lower percentage of macrophages compared to those obtained from healthy subjects, while the total number of recovered cells showed no differences. Induced sputa from healthy volunteers have been described to be poor in eosinophils, lymphocytes and epithelial cells, and rich in macrophages and neutrophils. To date, although repeated sputum induction has been suggested to have limited utility in serial assessment of neutrophilic airway inflammation due to the rise in the percentage of neutrophils over time,[32] the baseline mean values however range from 27.3% to 37.5%.[32–35] Eosinophils remain the most important and informative parameter particularly in asthma and COPD patients, while neutrophils can provide relevant information during infections as in patients with COPD.[36–38] On the contrary, owing to their low number, lymphocytes in IS are seldom used as a disease marker. However, with respect to ILD, D'Ippolito et al.[17] have shown that IS samples from newly diagnosed and untreated pulmonary sarcoidosis contains significant more cells, and particularly more lymphocytes and epithelial cells, when compared with sputa from healthy volunteers. To our knowledge, this is the first study investigating the inflammatory cell pattern in IS from patients affected by CTD with early clinical evidence of any lung involvement and in the absence of any evidence of respiratory infection. The concept of granulocyte traffic to the lung is not new, as neutrophils may play a critical role in the induction of tissue injury through the release of oxidant, proteolitic and pro-inflammatory mediators. In this issue, neutrophils and IL-8 levels have recently been found to be significantly increased both in alpha-1-antitrypsin PiMZ patients and in those affected by idiopathic pulmonary fibrosis, being inversely correlated with vital capacity in the latter.[39,40] Interestingly, in our series, sputum neutrophilic inflammation was detected even in cases with mild alterations of lung function parameters and no thoracic HRCT evidence of lung disease. Indeed, no correlation between sputum neutrophilia and lung function impairment or disease extension on HRCT was found. In addition and more surprisingly, even we found that $DLCO_{sb}$ was early compromised in the majority of tested patients (69%), there was not relation with sputum neutrophilia. Although larger and follow-up studies are further needed, this is however a quite interesting finding as IS may represent a valuable tool for detecting early abnormalities suggestive of subclinical lung involvement in CTD patients. Such an observation is even stronger as, in a similar manner, there is evidence that subclinical alveolitis may be present in a significant number of asymptomatic patients with both RA and SSc.[2,5,8] Finally, the fact that patients included in our study were undergoing anti-rheumatic treatment has also to be taken into consideration as most of them were receiving oral corticosteroids alone or in combination with immunosuppressive drugs. While the beneficial effect of inhaled corticosteroids on bronchial eosinophilic inflammation in asthmatic patients has been clearly established, results on sputum neutrophilia in asthma and COPD patients in response to anti-inflammatory therapies are contrasting.[41–43] To date, it has been recently reported that oral prednisolone, unlike inhaled budesonide, increases the recruitment of neutrophils in the airway submucosa of patients affected by mild asthma, while eosinophils, neutrophils, and levels of IL-8 and myeloperoxidase in IS do not change after treatment.[44] Although in our series we cannot exclude any treatment-related effect on the bronchial cell profile of studied patients, the observation that such a pattern significantly differed from that of healthy controls suggests that neutrophila may be a key hallmark of CTD-related airway inflammation. Again, as actually no more information is available in this issue, further efforts have to be focused in untreated patients.

Sputum induction primarily samples the more proximal airways containing neutrophil-rich secretions. To compare cell differentials in IS to those of the deep lung, BAL was carried out in 9 patients with thoracic HRCT evidence of interstitial involvement. As expected, we found that neutrophils were significantly increased in sputa than in BAL samples, where they were however represented at

increased levels (>10%) from baseline values (1–2%) in the 88% of patients, being more than 20% in two cases. In a similar fashion, a significant increase of neutrophils in IS than in bronchial biopsies and BAL samples was shown in sarcoidosis patients.[17] On the contrary, we found that the percentages of macrophages, lymphocytes and eosinophils were higher in BAL samples, while in agreement with previous observations the total cell number was lower than that recovered from IS.[10] Finally, we found a lack of correlation of the differential cell counts in the two different samples, which suggests that IS and BAL reflect different compartments of inflammation.

Aims of future studies could be to determine whether the non invasive assessment of airway inflammation adds significant information to that provided by the clinical history, lung function testing and thoracic HRCT data in CTD patients and whether this results in a relevant improvement concerning the patient's management. Although IS is a non-invasive diagnostic tool, as it was well tolerated and preferred to BAL by all patients in our study, no specific efforts have been carried out on the safety and functional effects of IS in patients with ILD and particularly in those with CTD-related lung involvement. In conclusion, IS procedure is far safer and easier to perform with no limitation to be frequently repeated. We suggest that it can be used as a complementary tool to lung function testing, HRCT and BAL in the early clinical assessment of CTD-related lung disorders. However, future studies are needed to better characterize airway inflammation in these patients and evaluate whether IS can replace more invasive procedures, i.e. BAL and HRCT, for disease monitoring and treatment follow-up.

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