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Inflammation and structural changes in the airways of patients with primary Sjögren's syndrome

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Abstract The present study aimed to compare the cellular pattern and structural changes in the airways of patients with primary Sjögren's syndrome (pSS) with healthy controls. Bronchial biopsy specimens were obtained from seven subjects with pSS and seven healthy controls. All the patients with pSS had increased bronchial responsiveness to methacholine. In the biopsies inflammatory cells, cytokine-producing cells, tenascin and laminin were visualized by immunostaining. Patients with pSS had a higher number of neutrophils and mast cells than healthy controls, while the number of eosinophils was similar in the two groups. The number of IL-8-positive cells was higher in pSS but the numbers of IL-4-and IL-5-positive cells were not significantly different between pSS and healthy controls. The numbers of T cells in patients with pSS were higher than in healthy controls, while the numbers of CD25-positive cells were similar to the healthy controls. The degree of epithelial integrity in patients with pSS was significantly lower than in the control group and the tenascin and laminin layers were significantly thicker in the pSS group. There was a correlation between the number of mast cells and the thickness of the tenascin and laminin layers in pSS. In conclusion, we found that the cellular pattern in the bronchial mucosa of patients with pSS displayed large numbers of neutrophils, mast cells and T-lymphocytes. These changes in inflammatory cell numbers seemed to relate to the observed increased epithelial damage and structural changes of the subepithelium. The structural findings, but not the pattern of inflammatory cells, are shared with atopic asthma and may relate to the increased bronchial hyper-responsiveness seen in both diseases.
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Keywords bronchoscopy; Sjögren's syndrome; inflammatory cells; cytokines; laminin; tenascin.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune inflammatory disease, characterized by lymphocytic and plasma cell infiltration, mainly in exocrine glands. Lung involvement is common in pSS (1) and the invasion of the lower respiratory tract by lymphocytic and plasma cell infiltrates in the mucous glands of the small airways gives rise to focal atelectasis and bronchiectasis, as well as lymphocytic pneumonitis and small airway disease (1). Patients with pSS, especially those who, in addition to glandular symptoms, suffer from extraglandular symptoms, frequently have bronchial hyper-responsiveness (BHR) (2–4) but are less reactive to indirect bronchial provocation agents such as adenosine monophosphate and cold air than patients with atopic asthma (4). In common with patients with asthma, patients with pSS often have increased levels of nitric oxide in exhaled air (5).

The reason for the occurrence of BHR in patients with pSS is not known. Gudbjörnsson *et al.* (3) speculated that it could be due to the presence of inflammatory cells in the airways, or that it could be related to xerotracheitis sicca and secondary to dry mucous membranes.

We hypothesized that the inflammation in the lungs, underlying BHR, of patients with pSS might involve similar mechanisms as that of patients with asthma. The aim of this study was therefore to compare the cellular patterns and structural changes in the airways of bronchial hyper-reactive patients with pSS with those found in healthy controls.

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Table 1. Patient characteristics (mean and range)

	Healthy controls (n=7)	Sjögren's syndrome (n=7)
Age (yrs)	30 (22–44)	51 (43–61)†
Sex (M/F)	2/5	0/7
FEV_1 (% pred)	98 (77–120)	85 (71–93)*
FVC (% pred)	95 (78–109)	86 (66–99)
PC_{20} (mg ml^{-1})	–	14.7 (0.7–32)

FEV_1: forced expiratory volume in 1); FVC: forced vital capacity, PC_{20}: provocation concentration that reduces FEV_1 by 20%. *$P < 0{\cdot}05$ and †0·01 compared with controls.

MATERIAL AND METHODS

Subjects

Bronchial biopsies were collected from 14 adults (Table 1), seven with pSS and seven healthy controls (Table 1). The participants formed part of a larger group included in an ongoing study of the pathophysiology of BHR (4–6).

The patients with primary pSS were being followed up as outpatients at the Department of Rheumatology, Uppsala University Hospital. The diagnosis of primary Sjögren's syndrome was based on the Copenhagen criteria (7). None of the patients were being treated with inhaled glucocorticosteroids, but two had inhaled β-agonists as needed and one had inhaled sodium cromoglycate. All the patients displayed an increased responsiveness to inhaled methacholine, defined by a provocative dose of methacholine causing a 20% fall in forced expiratory volume in 1 sec (FEV_1) $PC_{20} < 32$ mg (see below). All the patients were non-smokers and had been free from respiratory infections for at least 6 weeks prior to the study.

Controls were healthy subjects responding to a request for volunteers; none had asthmatic symptoms or was receiving anti-asthmatic treatment. All had a negative skin prick test. All were non-atopic and non-smokers.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the Faculty of Medicine at the University of Uppsala.

Laboratory procedures

Spirometry

Forced vital capacity (FVC) and FEV_1 were measured using a Masterlab Trans spirometer (Eric Jaeger AG, Würzberg, Germany).

Methacholine tests

An automatic, Spira Elektro 2 inhalation dosimeter (Respiratory Care Centre, Hameenlinna, Finland) was used (8). The provocation was continued by doubling the concentrations of methacholine until the FEV_1 had decreased by 20% or the highest concentration (32 mg ml^{-1}) was reached. Baseline values were measured after an inhalation of saline. The methacholine provocation was performed on average 5·8 months before the bronchoscopy. The mean PC_{20} values from these provocations are presented in Table 1. All the control subjects were negative to the methacholine provocation (PC_{20} values > 32 mg ml^{-1}).

Bronchoscopy

Patients were given 10 mg of diazepam (Stesolid®, Dumex, Copenhagen, Denmark) orally and 0·5 mg of atropine (Atropine, NM Pharma, Stockholm, Sweden) subcutaneously 30 min before the investigation. The upper airways were anaesthetized with lidocaine hydrochloride (Xylocaine, Astra, Södertälje, Sweden). Using a flexible fibre bronchoscope (Olympus P 20D) with a FB 15C 2·0 mm forceps (Olympus, Tokyo, Japan), two biopsies were taken in the right lung in the superior lobar bronchus immediately after the division from the main bronchus. The specimens were examined immediately under a light microscope to ensure the presence of a complete mucosa and processed as described below.

Immunohistochemistry

Frozen sections

One of the bronchial biopsy specimens taken from the upper lobe was frozen immediately and rapidly in propane cooled down with liquid nitrogen.
The method used in the immunohistochemical analysis of the biopsies has been described in detail in a previous paper (6).

Monoclonal antibodies

The eosinophils, neutrophils and mast cells were identified with monoclonal antibodies. To ensure a reliable

count of neutrophils, two different antibodies, human neutrophil lipocalin (HNL) (Pharmacia & Upjohn Biosensors, Uppsala, Sweden) and myeloperoxidase (MPO) (Dako, Glostrup, Denmark), were used for identification.

The monoclonal antibodies EG1 (eosinophil cationic protein; ECP) and EG2 (eosinophil cationic protein/eosinophil protein-X; ECP/EPX) (Pharmacia & Upjohn Diagnostics AB) were used for the identification of eosinophils. The anti-tryptase antibody 1, AA1 (Dako), was used for the identification of mast cells. For the localization of interleukins 4 and 5 (IL-4 and IL-5), mouse anti-human IL-4 and rat anti-human IL-5 were used. Both antibodies came from PharMingen (San Diego, CA, U.S.A.). Monoclonal mouse anti-IL-8 (Pharmacia & Upjohn, Diagnostics AB) was used on frozen sections at a concentration of 0·002 mg ml^{-1}. A panel of monoclonal antibodies including anti-CD3, CD4, CD8 and CD25 (IL-2 receptor) was used to identify various types of T-lymphocyte (Dako).

Tenascin and laminin in cryosections

Frozen sections were thawed and fixed in acetone at -20°C for 10 min prior to the addition of the monoclonal antibodies to tenascin and laminin (Dako).

Plastic-embedded tissue for morphology

For morphological studies, light microscopy was carried out on glycol methacrylate (GMA), (Agar Aids, Stansted, U.K.) embedded tissue as described previously by Britten (9). Sections with a thickness of 2 μm were cut with glass knives on an LKB Historange Microtome (LKB Instruments, Bromma, Sweden).

Microscopic evaluation of sections

All the specimens were coded and examined by the microscopist without any knowledge of the diagnosis. The number of labelled cells in the tissue was counted manually at a magnification of $\times$ 40. One field of approximately 0·8 mm^2 was selected a random. This field did not include glands and muscle cells. One field was analysed in each section.

The epithelial integrity of the bronchial wall of the subjects from the different groups was assessed by light microscopy in plastic sections stained with Mayer's haematoxylin. The total length of the basement membrane and the length of intact epithelium in the section through each biopsy were determined. Epithelial integrity was defined as the length of basal membrane with intact epithelium divided by the total length of the membrane.

The measurement of the thickness of the tenascin and laminin layers (in mm) was performed in 4-μm thick immunolabelled frozen sections using a $\times$ 40 objective and a computerized image analysis system as described above.

Statistics

All the statistics were calculated using non-parametric tests. Comparisons between the two groups were performed with the Mann–Whitney *U*-test. For correlations within a group, Spearman's rank correlation test was used. A *P*-value of $<0·05$ was regarded as statistically significant.

RESULTS

The patients with pSS were significantly older than the healthy controls. There was also significantly difference of FEV_1 in patients with pSS compared with healthy controls (Table 1).

Inflammatory cells and cytokines

The number of neutrophils (HNL- and MPO-positive cells) was higher in patients with pSS than in healthy controls, but there was no significant difference in the numbers of eosinophils (EG1- and EG2-positive cells). The numbers of mast cells were increased in patients with pSS compared to the control group (Table 2).

The total number of T-lymphocytes and the numbers of CD3-, CD4-, CD8-positive cells were increased in the pSS group (Table 2), whereas the numbers of CD25-positive cells were similar in pSS and healthy controls (Table 2).

The numbers of cells expressing cytokines in the two groups are shown in Table 2. IL-4-positive cells and IL-5-positive cells were similar in pSS and healthy controls, whereas patients with pSS had significantly higher numbers of IL-8-expressing cells (Table 2). IL-8 was mainly found in bronchial epithelial cells and neutrophils.

Correlation between inflammatory cells

In the patients with pSS the numbers of MPO-positive cells were correlated significantly with the numbers of IL-8- (r=0·83) and CD4-positive cells (r = 0·75) ($P < 0·05$). A significant positive correlation was also found between HNL- and IL-8-positive cells in the pSS group (r = 0.88, $P < 0·01$).

Epithelial damage and structural changes

Patients with pSS had lower epithelial integrity than controls (Fig. 1). In the damaged area, the cylindrical ciliated epithelial cells were absent, while the layer of cuboidal

Table 2. Number of granulocytes, *T*-lymphocytes and cells expressing cytokine in bronchial biopsies (median and inter-quantity range)

	Healthy controls (n=7)	Sjögren's syndrome (n=7)
EGI	0 (0–1)	33 (0–49)
EG2	0 (0–1)	22 (6–56)
HNL	24 (12–25)	133 (122–200)‡
MPO	28 (26–30)	267 (155–333) ‡
AAI	6 (3–7)	56 (50–61) ‡
CD3	14 (11–15)	280 (240–360) ‡
CD4	8 (6.5–10)	190 (175–280) ‡
CD8	5 (5–6.5)	80 (62–86) ‡
CD25	0 (0–0)	0 (0–6)
IL-4	0 (0–0.5)	0 (0–5)
IL-5	0 (0–1)	0 (0–6)
IL-8	0 (0–5)	48 (45–56) ‡

EG: eosinophil granulocyte; HNL: human neutrophil lipocalin; MPO: myeloperoxidase; AAI: anti-tryptase antibody 1 ; CD: clusters of differentiation; IL: interleukin.
Results are given as positive cells mm^{-2}.
‡$P < 0{\cdot}001$ compared with controls.

basal cells was often intact. Neutrophils in pSS were frequently found in the areas of epithelial damage (Fig. 2).

The tenascin and laminin layers were thicker in patients with pSS as compared to the healthy controls (Fig. 3).

Correlations between inflammatory cell numbers and epithelial integrity or structural changes

In pSS epithelial integrity was negatively correlated to the numbers of eosinophils, CD3- and CD4-positive T-lymphocytes, neutrophils and IL8-positive cells (Table 3). Positive correlations were found between the numbers of mast cells and the thickness of either the tenascin or the laminin layer (Table 3).

DISCUSSION

This study has shown major changes in the histopathologic characteristics of the lungs of patients with pSS and increased responsiveness to metacholine. These patients were characterized by high numbers of neutrophils, mast cells and T-lymphocytes in their bronchial mucosa, concomitant with a high number of IL-8-positive cells. In contrast, the numbers of eosinophils seemed normal, as did the numbers of cells staining positive for IL-4 and IL-5. Our study also indicated that patients with pSS in

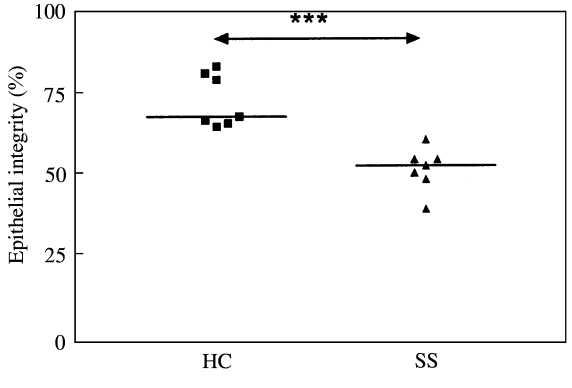

Fig. 1. A comparison of the epithelial integrity of the airway wall in healthy controls (HC), and patients with primary Sjögren's syndrome (pSS).

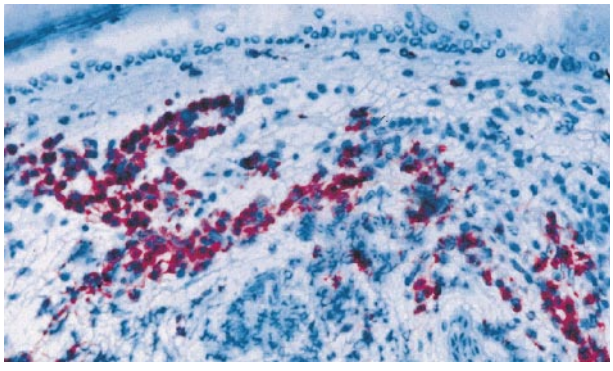

Fig 2. Resin (plastic) sections of a bronchial biopsy with HNL. Neutrophils accumulation in areas of reduced epithelial integrity is shown in a specimen from a patient with pSS. Mayer's haematoxylin. Original magnification × 320.

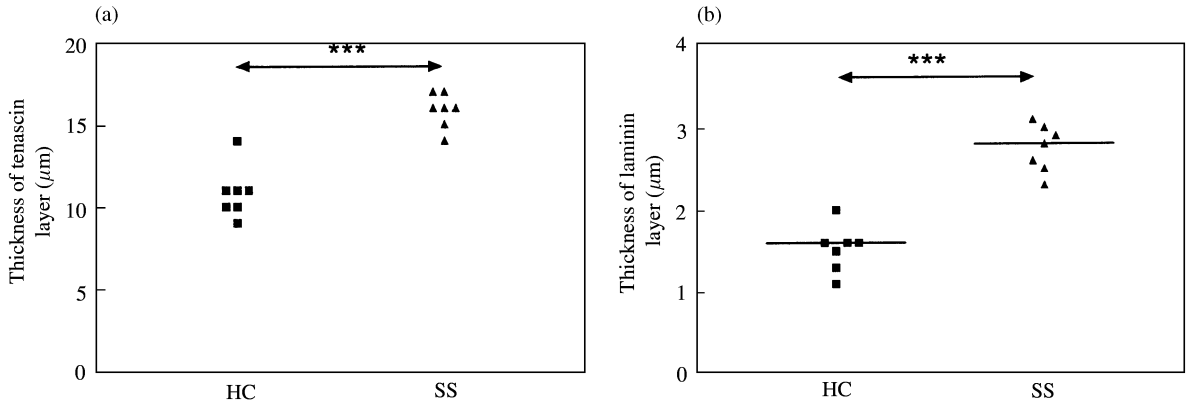

Fig. 3. Structural changes in the airways of the different groups as indicated by the thickness of the tenascin (a) and laminin (b) layers in the subepithelium of healthy controls (HC), and patients with primary Sjögren's syndrome (pSS).

comparison with healthy controls have increased epithelial damage and thickness of the tenascin and laminin layers in the airways.

In a previous study we investigated the inflammatory and structural changes in the bronchial mucosa of another group of patients with increased bronchial responsiveness to metacholine, i.e. bronchial asthma (6). A rationale for comparing such patients with pSS was that previous studies have shown that patients with pSS share some characteristics with asthmatics, such as a high prevalence of airway symptoms and bronchial hyperresponsiveness (2, 3). In the same group of patients as in this study, it was found that both patients with pSS and atopic asthma are associated with increased levels of exhaled NO (10), while patients with pSS were less likely to react to indirect bronchial provocation stimuli than the atopic asthma group (4). The results of this bronchoscopy study confirmed that there are distinct similarities between patients with pSS and atopic asthma regarding structural changes, but there are also clear differences in involvement of inflammatory cells as will be discussed below. It should, however, be emphasized again that the patients of this study were selected because of their hyper-responsiveness to metacholine. This be kept in mind when comparing of our data with those of others studying inflammatory and structural changes in pSS.

The large numbers of neutrophils found in bronchial biopsies in patients with pSS are in accordance with those studies reporting an increased number of neutrophils in bronchial alveolar lavage (BAL) (11). The pSS biopsies contained only a few eosinophils, which also is in line with the results of Papirin *et al*. who used the haematoxylin–eosin method and found no significant difference in eosinophil numbers between patients with pSS and controls (12). Other authors, however, have found large numbers of

Table 3. Correlation (*r*-values) between inflammatory cell numbers and structural changes in patients with Sjögren's syndrome

Cells	Epithelial integrity	Tenascin layer	Laminin layer
EG1-positive	−0·76*	0·54	−0·34
EG2-positive	−0·83*	0·49	0·02
MPO-positive	−0·76*	0·02	−0·36
HNL-positive	−0·83*	0·59	0·43
AA1-positive	−0·56	0·80†	0·56*
IL-4 − positive	−0·35	0·46	0·31
IL-5-positive	−0·34	−0.46	0·32
IL-8-positive	−0·88‡	0·54	0·04
CD3-positive	−0·70*	0·59	0·21
CD4-positive	−0·86†	0·50	0·18
CD8-positive	−0·52	0·56	−0·18
CD25-positive	−0·20	0·46	0·61

EG: eosinophil granulocyte; MPO: myloperoxidase; HNL: human neutrophil lipocalin; AAI: anti-tryptase antibody 1; CD: clusters of differentiation; IL: interleukin.
*$P < 0{\cdot}05$, †$P < 0{\cdot}01$, ‡$P < 0{\cdot}001$.

eosinophils in the BAL from pSS (13). The numbers of mast cells were increased in patients with pSS.

The role of airway mast cells in Sjögren's syndrome is not known, but Skopouli *et al.* (14) showed that mast cells in the salivary glands of patients with primary pSS are strongly associated with fibrosis. This may indicate that the increased number of mast cells in the bronchial mucosa in pSS plays an important role in the airway pathology of this disease; a notion that was further suggested by the close association between mast cell numbers and the thickness of the laminin and tenascin layers. We found a similar close association in atopic asthma (6), which further emphasizes a likely role of mast cells in these processes.

A common feature of all the organs affected by pSS is a dense lymphocyte infiltrate (15). It is thought that this infiltrate may cause functional changes in the affected organs and may be responsible for the diverse clinical features of this syndrome. In the present investigation, large numbers of lymphocytes (CD3-, CD4-, CD8-positive cells) were found in patients with pSS compared with healthy controls. Gudbjörnsson *et al.* (16, 17) have previously demonstrated increased levels of lymphocytes in BAL fluid in pSS. Coll *et al.* (18) have shown high IL-2R (CD25) expression in epithelial cells in the salivary glands of patients with pSS. In the present study we did not, however, find any expression of CD25 in the epithelial cells or other cells in bronchial biopsies from these patients.

Our investigation showed a large number of IL-8-positive cells in pSS. In addition to lymphocytes, IL-8 was predominantly localized in bronchial epithelial cells and neutrophils, whereas in patients with atopic asthma eosinophils seemed to the major containing cell (6). We did not find many IL-4- and IL-5-positive cells in patients with pSS. This result is in agreement with Konttinen (19), indicating that inflammation in pSS is not driven by Th2-cells.

Damage to the bronchial epithelium has often been reported in bronchial biopsy specimens of patients with asthma (6, 20). Previously we showed that such damage was a hallmark of atopic asthma, since it was not observed in non-atopic asthma. In atopic asthma the epithelial damage was correlated to the presence of eosinophils in the mucosa (6). In this study on pSS we also found a reduced epithelial integrity, although not to the same extent as in atopic asthma. To our knowledge, increased damage of bronchial epithelium has not been described before in patients with pSS. Suzuki *et al.* (21), however, reported increased epithelial damage in the eye of pSS.

The possible mechanism of damage of the bronchial epithelium in pSS seems to be much more complex than in atopic asthma and involve several cells, since we found correlations to both neutrophils, eosinophils and T-lymphocytes. Both the eosinophil and the neutrophil can release large amounts of toxic oxygen radicals and a variety of granule proteins with cytotoxic and potent enzymatic properties such as ECP, MBP, neutrophil elastase, myeloperoxidase (MPO), lactoferrin and lipocalins, which could take part in this damage. The correlations to T-lymphocytes could indicate that these cells too play a role in the direct damage of the epithelial cells. A more likely explanation for these relationships, however, is the fact that activated lymphocytes may be involved in the attraction and activation of eosinophils and neutrophils in pSS. Other possible mechanisms behind the epithelial damage observed in pSS include the dryness of the mucous membrane, which could make the epithelium more vulnerable to damage. The shedding of ciliated epithelial cells from basal cells may be caused by the release of TNFα by macrophages or lymphocytes, the release of proteases and other enzymes (22, 23) by several cell types, or alternatively by submucosal oedema.

Thickened layers of laminin and tenascin are other hallmarks of atopic asthma. The layers of laminin and tenascin in pSS were clearly thicker than in healthy subjects, but less so than in atopic asthma (6). As noted above our previous results on atopic asthma suggested a role of mast cells in the process (6). It is therefore intriguing to find a similar relationship in pSS, since this might suggest a causal relationship. It is well known that mast cells bind to extracellular structures such as laminin (24) and the activities of mast cells have been implicated in the development of fibrotic disorders (25).

A drawback with our study was that we have not matched the patient group and control group for age and sex. The patient group was on average 20 years older than the control group and we can therefore not totally exclude that some of the differences reported here may be related to the age difference. We are not aware if the effect of ageing on airway inflammation has been studied in detail. In previous investigations we have, however, not found any significant correlations between age and indirect measures of airway inflammation such as exhaled NO or serum levels of eosinophil cationic protein in healthy subjects (10, 26).

In conclusion, we found that the cellular pattern in the bronchial mucosa of patients with pSS displayed large numbers of neutrophils, mast cells and T-lymphocytes. These changes in inflammatory cell numbers seemed to relate to the observed increased epithelial damage and structural changes of the subepithelium. The structural findings, but not the pattern of inflammatory cells, are shared with atopic asthma and may relate to the increased bronchial hyper-responsiveness seen in both diseases.

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