Interleukin 1β and tumour necrosis factor α secreting cells are increased in the peripheral blood of patients with primary Sjögren’s syndrome

P Willeke, H Schotte, B Schlüter, M Erren, H Becker, A Dyong, E Mickholz, W Domschke, M Gaubitz

Objective: To study systemic alterations of cytokine secreting peripheral blood mononuclear cells (PBMC) in primary Sjögren’s syndrome (pSS) and their relation to common clinical and immunological manifestations of this disease.

Methods: PBMC spontaneously secreting tumour necrosis factor α (TNFα), interleukin 1β (IL1β), and interleukin 6 (IL6) were assessed by enzyme linked immunospot (ELISPOT) analysis in a cohort of 31 patients with pSS fulfilling the modified European classification criteria. Nineteen healthy volunteers served as controls. ELISPOT results were correlated with glandular and extraglandular manifestations and autoantibody titres—that is, rheumatoid factor (RF) isotypes, anti-La/SS-A, anti-La/SS-B as determined by an enzyme linked immunosorbent assay (ELISA) technique.

Results: The number of TNFα and IL1β secreting cells was significantly higher in patients than in controls. No differences were detected in the number of IL6 secreting PBMC. Patients with recurrent parotid swelling (RPS) had a significantly increased number of IL1β secreting PBMC. Moreover, the number of IL1β secreting PBMC correlated with the disease duration (r=0.479; p=0.01) and with the concentration of IgM RF (r=0.63; p=0.01) and IgG RF (r=0.42; p=0.05). Other autoantibodies did not correlate with cytokine secreting PBMC.

Conclusion: The increased systemic secretion of IL1β and TNFα in patients with pSS points to a pathogenic impact of these cytokines in this autoimmune disease. In particular the correlation of IL1β secreting PBMC with RPS and RF production indicates that IL1β is a crucial regulator in the development of local and systemic disease manifestations.

Primary Sjögren’s syndrome (pSS) is an autoimmune disorder characterised by chronic lymphocytic infiltration of exocrine glands leading to keratoconjunctivitis sicca and xerostomia.

Although the pathophysiology of pSS is not yet fully understood, there is increasing evidence that tumour necrosis factor α (TNFα), interleukin (IL)1β, and IL6 are important mediators of the autoimmune response and have a potential role in mediating tissue destruction of salivary and lacrimal glands.1 2

Several studies have reported high levels of mRNA expression of these proinflammatory cytokines in the salivary glands of patients with pSS.3 4 IL6 was also found at increased levels in the saliva.5 Increased IL1 and IL6 levels have been reported in tear fluid.6 11 Increased serum levels of TNFα have been detected in the serum of patients with pSS with La/SS-B antibodies.6 However, conflicting data on systemic IL6 levels in patients with pSS have been reported.2 11

Most studies on cytokines in pSS focused on the local pattern of cytokine production in the labial salivary gland (LSG). A central aim of our study was to determine systemic alterations of the production of proinflammatory cytokines by peripheral blood mononuclear cells (PBMC). Moreover, we wanted to determine whether abnormalities in the cytokine production are associated with the production of specific autoantibodies (that is, anti-La/SS-A, anti-La/SS-B, and rheumatoid factor (RF) idiotypes) or with different clinical findings.

To examine these issues an enzyme linked immunospot (ELISPOT) assay was performed to determine the secretion of TNFα, IL1β, and IL6 at the single cell level ex vivo.

Patients and healthy controls

Thirty one patients with pSS (30 female, one male) aged 30–75 years were included. The diagnosis was based on the recently revised European criteria.3 In 18 patients an LSG biopsy had been performed previously, demonstrating a focus score $\geq$ 1. Although several patients had had an LSG biopsy for diagnosing the disease in the past, this invasive technique was inapplicable as a routine follow up procedure in our patients. Exclusion criteria were concomitant severe diseases or infections. Patients taking corticosteroids, disease modifying antirheumatic drugs, or drugs that potentially diminish exocrine gland function were also excluded.

Nineteen age matched healthy volunteers served as controls. Patients and controls gave informed consent.

The disease duration was defined as the time which had elapsed since the onset of sicca syndrome. Parotid swelling or conjunctivitis was defined as recurrent if occurring more than three times in the recent year.

Cell isolation and culture

Peripheral blood (20 ml) was drawn into a heparinised sampling tube by venepuncture between 9 00 am and 10 00 am in order to prevent the bias due to circadian alterations of cytokine levels that has been reported.6 PBMC were separated by density gradient centrifugation over Ficoll-Hypaque (density 1.077, Biochrom KG, Berlin, Germany). Culture of PBMC was performed as previously described.7

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; ELISPOT, enzyme linked immunospot; ESR, erythrocyte sedimentation rate; IL, interleukin; LSG, labial salivary gland; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; pSS, primary Sjögren’s syndrome; RF, rheumatoid factor; RPS, recurrent parotid swelling; TNFα, tumour necrosis factor α
**Human cytokine ELISPOT assay**

Ninety-six well nitrocellulose backed microtiter plates (Millipore, Bedford, USA) were coated with 10 μg/ml of monoclonal antibodies against TNFα, IL1β, and IL6 (Endogen, Boston, USA) and incubated overnight at 4°C. Wells were washed with phosphate buffered saline (PBS)-Tween and blocked with 200 µl of PBS containing 5% bovine serum albumin (BSA) at 37°C for 30 minutes. The wells were washed again extensively with PBS-Tween. Serial dilutions of triplicate cell suspensions, starting with 10⁶ cells/well were incubated on the cytokine coated plates in a humidified atmosphere with 5% CO₂ at 37°C for 18 hours. The plates were incubated on the cytokine coated plates in a humidified atmosphere with 5% CO₂ at 37°C for 18 hours. The plates were washed with phosphate buffered saline (PBS)-Tween and overlaid with 500 ng/ml of biotinylated anti-TNFα, anti-IL1β, and anti-IL6 antibodies (Endogen) for two hours. After additional washes 50 µl/well of avidin-alkaline phosphatase (Sigma St Louis, MO, USA) was added in a dilution of 1:300 with 1% BSA. Plates were incubated at room temperature for two hours before washing in PBS-Tween.

The cytokines secreted by single cells were visualised by the addition of the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma). The colorimetric reaction was halted after five minutes by rinsing three times with deionised water.

Spots were automatically counted with an electronic computer assisted imaging system (Autoimmun Diagnostika GmbH, Straßberg, Germany), which has been shown to be more valid and precise than manual microscopic counting of the immunospots. The precision was generally acceptable (coefficient of variation for repeated measurements <15% for IL1β, 24% for IL6, and 30% for TNFα).

**Other laboratory tests**

Routine laboratory measurements (that is, erythrocyte sedimentation rate (ESR), C reactive protein, white and red blood cell count, serum creatinine, total protein, and electrophoresis) were obtained at the same time as the ELISPOT assay.

Antinuclear antibodies were demonstrated by indirect immunofluorescence on HEp-2 cell as substrate (Innogenetics, Heiden, Germany). Antibodies against U1-snRNP (68 kDa, A, C), Ro/SS-A (52 kDa, 60 kDa), La/SS-B, SCI-70, CENP-B, Jo-1, and native DNA were measured by semiquantitative enzyme linked immunosorbent assay (ELISA; Pharmacia Upjohn, Freiburg, Germany).

RF was determined by the Waaler-Rose and latex fixation tests (Dade Behring, Schwalbach, Germany). The levels of IgA, IgM, and IgG RF as well as anti-Ro/SS-A and anti-La/SS-B of IgG isotype were determined by an ELISA technique (Pharmacia Upjohn, Freiburg, Germany). Serum concentrations of IgG, IgA, IgM and complement levels (that is, C3 and C4) were measured by nephelometry (Dade-Behring, Schwalbach, Germany).

**Statistical analysis**

The Mann-Whitney U test was used for unpaired samples. For paired samples the Wilcoxon-test was employed. A Spearman correlation test was used to correlate clinical data with laboratory results. A p value <0.05 was considered as significant.

**RESULTS**

Table 1 summarises the clinical characteristics of patients and controls. All patients had a positive testing for anti-Ro/SS-A or anti-La/SS-B.

**Frequency of cytokine secreting PBMC in patients with pSS**

In patients with pSS the number of cells spontaneously secreting TNFα and IL1β was increased in comparison with controls (p<0.01; fig 1) in contrast with IL6 secreting PBMC.

**Association of cytokine secreting PBMC with clinical features**

In patients with recurrent parotid swelling (RPS) the number of IL1β secreting PBMC was significantly higher than in patients without RPS (p<0.05) and normal controls (p<0.01) (fig 2).

Further, we found a clear correlation between the disease duration and the number of IL1 secreting PBMC (r=0.479; p<0.01), whereas no correlation with patients’ age was found.

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**Table 1** Characteristics of patients and healthy controls. Results are shown as No (%) except where stated otherwise

<table>
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<tr>
<th></th>
<th>Patients (% of total)</th>
<th>Healthy controls</th>
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<tr>
<td>Number</td>
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</tr>
<tr>
<td>Sex (M/F)</td>
<td>1/30</td>
<td>6/13</td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>49.7 (13.1)</td>
<td>47.3 (12.5)</td>
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<td>Disease duration (years), mean (SD)</td>
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<td>Biopsy</td>
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<td>Glandular manifestations</td>
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<tr>
<td>Recurrent parotid swelling</td>
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<td>0</td>
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<td>Recurrent conjunctivitis</td>
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<td>Extranglandular manifestations</td>
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<td>Peripheral neuropathy</td>
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<td>Thyroiditis</td>
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<td>RF (Waaler-Rose test )</td>
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<tr>
<td>ANA</td>
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<td>Anti-Ro antibodies</td>
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<td>Anti-La antibodies</td>
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**Figure 1** Number of TNFα, IL1β, and IL6 secreting PBMC in patients with pSS and healthy controls. The data of the box plots are given as median plus the 25th/75th centiles. Bars represent the total range of values.
PBMC in patients with pSS.

There was a significant correlation between the number of cytokine secreting PBMC with laboratory parameters. There was a significant correlation between the number of IL1β secreting PBMC and IgM RF titres ($r_s=0.63$; $p<0.01$); and a weaker correlation with IgG RF titres ($r_s=0.42$; $p<0.05$). Also the result of the latex fixation test and the amount of IL1β secreting PBMC correlated significantly ($r_s=0.44$; $p<0.05$). The number of TNFα or IL6 secreting PBMC did not correlate with RF titres.

No correlation was found between the number of cytokine secreting PBMC and anti-Ro/SS-A or anti-La/SS-B antibody titres or other laboratory parameters (that is, ESR, C reactive protein, complement levels, blood cell counts, serum creatinine, total protein, and electrophoresis).

**DISCUSSION**

Most studies on the role of cytokines in pSS have focused on the local cytokine expression at sites of inflammation. In this study we performed an ELISPOT assay to detect cells actively secreting proinflammatory cytokines in the peripheral blood of patients with pSS in order to determine systemic alterations in this disease. Our data demonstrate for the first time an increased prevalence of TNFα and IL1β secreting PBMC in patients with pSS.

TNFα and IL1β have been shown to stimulate the production of collagenases. Both cytokines enhance the secretion of matrix metalloproteinase 2, synthesised by acinar cells, and thus may promote disruption of acinar cell architecture. Data from animal models and pilot studies with TNFα blocking agents suggest that these agents are beneficial in the treatment of pSS, which emphasises the potential role of TNFα as a key cytokine in the pathophysiology of pSS. We suggest that the systemic secretion of these cytokines may have a role in the pathogenesis of this autoimmune disease.

In our study an increase in IL1β secreting PBMC was associated with RPS. As previously reported by Daniels, RPS is associated with a high focus score in biopsy specimens from the LSG of patients with pSS. The inflamed exocrine glands are the major site of lymphocytic hyperactivity in pSS.

We suggest that the systemic increase of IL1β secreting PBMC may promote local inflammatory processes in exocrine glands of patients with pSS and thus may serve as a marker of disease activity, although a correlation with a current focus score of LSG biopsies has not been performed. Further investigations are needed to evaluate this issue.

**REFERENCES**

receptor (PEG sTNF-R1) prevents development of Sjögren’s syndrome and diabetes in the NOD mouse model [abstract]. Arthritis Rheum 2000;42(suppl 9):403.


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