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Concise Report

Cytokine and autoantibody profiling related to histopathological features in primary Sjögren's syndrome

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Objective. To investigate a potential correlation between circulating cytokine and autoantibody levels and histopathological features in subgroups of patients with primary SS (pSS).

Methods. Minor salivary gland biopsies from a cohort of 141 patients fulfilling the American–European consensus classification criteria for pSS were re-examined and grouped according to focus score (FS) and germinal centre (GC) status; serum samples were analysed for autoantibodies, chemokines and cytokines.

Results. Of the 115 available biopsies, 18 (16%) lacked characteristic focal mononuclear cell infiltrates [FS <1 (FS$-$)] but patients were positive for Ro/SSA and/or La/SSB. IL-17, IL-1RA, IL-15, macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, IFN-α and IL-4 levels were significantly increased in the 27 (23%) patients with ectopic GC formation (GC+) in the salivary glands compared with the GC$-$ patients ($n=70$). In addition, minor differences in cytokine levels were found when comparing age groups.

Conclusion. Degenerative changes observed in the minor salivary glands of patients with pSS may represent 'burned out' inflammation. The elevated levels of IL-4 found in these patients may influence the reduced salivary flow observed in GC+ patients. Increased titres of Th17-associated cytokines, IL-17, IL-1β and the IL-23 subunit IL-12p40, may indicate a higher activity of these cells in GC+ patients. Differences in cytokine levels may be utilized when sub-grouping the SS patients into disease phases and may consequently have implications for treatment.

Key words: Adiopocytes, Atrophy, Autoantibodies, Cytokines, Degenerative changes, Fibrosis, Inflammation, Salivary glands.

Introduction

SS is a complex autoimmune rheumatic disease characterized by the presence of mononuclear cell infiltration of exocrine tissues and autoantibodies against the RNP particles Ro/SSA and La/SSB [1]. Primary SS (pSS) occurs alone whereas secondary SS (sSS) occurs in association with other autoimmune rheumatic diseases. The diagnosis of pSS is based on distinctive subjective and objective features of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). For a definite diagnosis, the patients need to fulfil at least three subjective and/or objective requirements, in addition to positive serology for anti-Ro/SSA and/or anti-La/SSB, or focal mononuclear cell inflammation corresponding to focus score (FS) $\geqslant 1$ [2].

The histopathological findings in glandular biopsies from patients with SS are a progressive focal infiltration of mononuclear lymphoid cells [3]. This correlates largely to the reduced salivary secretion, but a direct association between the degree of lymphoid infiltration and exocrine dysfunction is not always obvious [4]. Mechanisms leading to attraction and accumulation, and the biological role of the infiltrating cells remain unknown. Focal chronic inflammation and formation of ectopic germinal centre (GC)-like structures have been thoroughly investigated in patients with pSS [5–8], but features of seropositive pSS patients lacking sufficient inflammation to fulfil FS remains to be further elucidated.

Cytokines are important mediators in inflammation and immune reactions and over the past years the role of specific cytokines in SS has been extensively studied [9]. Recent reports suggest up-regulation of IL-17 and IL-23 in SS, but an association with specific clinical manifestations has not been established [10].

The overall aim of this study was to investigate histopathological and serological features in subgroups of patients with pSS.

Materials and methods

Patients and tissue samples

A cohort of 141 patients with pSS [2] recruited from the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway, were investigated in this study. Informed and written consent was obtained from all patients who participated in this study.

Formalin-fixed and paraffin-embedded haematoxylin and eosin (H&E)-stained sections were available from 115/141 patients. In 97/115 patients, minor salivary gland tissue inflammation corresponding to FS $\geqslant 1$ (FS+) was determined (Fig. 1A and B). FS describes the number of inflammatory cell foci containing at least 50 mononuclear cells per 4 mm^2. In the remaining cases ($n=18$) FS was not fulfilled (FS$-$; Table 1). The study was approved by the Committee of Ethics at the University of Bergen (145/96-44.96 and 242.06).

Evaluation of minor salivary gland tissue

Ectopic GC formation. FS+ patients ($n=97$) were screened for the presence of ectopic GC$-$ like structures; 70 (72%) were considered GC$-$ and 27 (28%) GC+ patients. A GC-like structure was defined as a well-circumscribed chronic inflammatory cell infiltrate consisting of at least 50 mononuclear cells presenting with a densely packed dark zone and a less densely packed light zone (Fig. 1C), within otherwise normal salivary gland epithelium,

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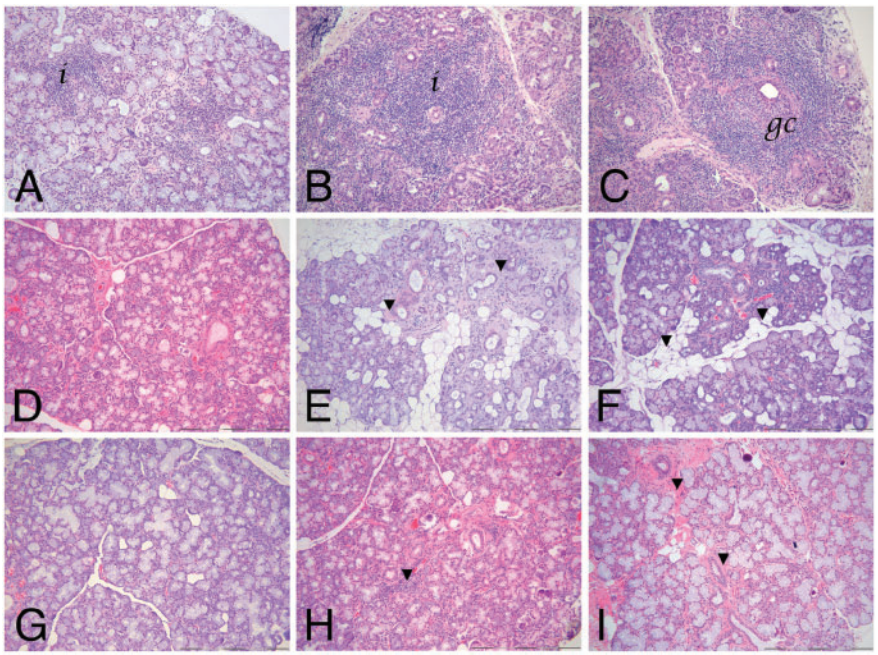

FIG. 1. Focal mononuclear cell infiltrates corresponding to FS $\geqslant$ 1 were observed in the minor salivary glands in 115/141 patients. (**A**) Two separate focal mononuclear cell infiltrates (i) in minor salivary gland tissue from a patient with FS 3. Note otherwise normal minor salivary gland tissue surrounding the focal infiltrates. (**B**) Two confluent focal mononuclear cell infiltrates (i) in minor salivary gland tissue from a patient with FS 7. (**C**) Minor salivary gland tissue from the same patient as in (B) showing an infiltrate with GC-like structure within otherwise normal minor salivary gland tissue. Degenerative changes were observed in the minor salivary gland tissue not characterized by focal mononuclear cell infiltrates fulfilling FS 1 (FS−). (**D**) Chronic inflammation ($n=5$). Diffuse/even distribution of lymphoid cells but lacking focal infiltrates. (**E**) Atrophy of glandular tissue/acinar epithelium ($n=10$) as indicated by the arrowheads. (**F**) Fatty cell degeneration ($n=1$) as indicated by arrowheads. (**G**) Normal morphology ($n=2$). (**H**) Focal infiltrates were present in 8/18 (44%) biopsies, but were either sparse or contained less than 50 cells, consequently not fulfilling FS 1 (FS−). (**I**) Interstitial and/or periductal fibrosis was observed in 13/18 (72%) biopsies. Arrowheads indicate interstitial and/or periductal fibrosis. Fibrosis coincided with previously diagnosed chronic inflammation ($n=3$), atrophic minor salivary gland tissue ($n=8$), fatty cell degeneration ($n=1$) and non-inflamed salivary gland tissue ($n=1$). Upon re-evaluation, varying degrees of fatty cell degeneration were observed in 10/18 (56%) patients. Fibrosis was observed in 8/10 (80%) of these biopsies.

TABLE 1. Comparison of patients in the subgroups FS−, FS+, GC− and GC+ of pSS

			FS+	
Clinical features	FS−	FS+	GC−	GC+
Number of patients, *n*	18	97	70	27
Mean age, years	49 ± 3	51 ± 1	53 ± 1	46 ± 2[#]
RF $\geqslant$128	0/18[‡]	14/79	7/60	7/19
Ro/SSA+	16/2[‡]	40/53*	26/41	14/12
La/SSB+	6/12	22/71	13/54	9/17
Ro/SSA + or La/SSB+	18/0[‡,a]	44/49*	28/39	16/10
Ro/SSA + and La/SSB+	4/14	18/75	11/56	7/19
Unstimulated whole saliva	1.68 ± 0.22	2.03 ± 0.25	2.48 ± 0.33	0.95 ± 0.23[#,§]
Salivary secretion $\leqslant$1.5 ml/15 min	9/9[‡]	51/37	30/32	21/5
Mean FS	NA	2.42 ± 0.16	2.11 ± 0.17	3.22 ± 0.33[#]

Data presented as mean ± S.E.M; fractions indicate the number of patients with and without the observed phenomenon. Not all clinical data were available for all patients. [‡]A significant ($P<0.05$) difference when comparing the groups GC+, GC− and FS−. *A significant ($P<0.05$) difference when comparing the groups FS− and FS+. [§]A significant ($P<0.05$) difference when comparing the groups GC+ and FS−. [#]A significant ($P<0.01$) difference when comparing the groups GC+ and GC−. [a]Necessary to fulfill pSS criteria [2].

as previously described by Jonsson *et al.* [7]. This was in comparison with conventional focal infiltrates where such organization of lymphoid cells was not observed (Fig. 1A and B).

Other histopathological changes. Minor salivary gland tissue sections from the FS− patients were re-evaluated with focus on chronic inflammatory and degenerative changes such as interstitial chronic inflammation, atrophy of glandular/acinar epithelium, fatty cell degeneration, and interstitial and/or periductal fibrosis (Fig. 1D–I).

Multiplex bead immunoassay

Human Cytokine 25-Plex kit (cat. # LHC0009, Invitrogen, Carlsbad, CA, USA) was used for cytokine quantification in serum, and QUANTA Plex™ SLE Profile 9 immunoassay (cat. #708910, San Diego, CA, USA) was used for autoantibody quantification in serum as recommended by the manufacturers. Plates were analysed with a Luminex 100™ instrument with StarSection software (Applied Cytometry Systems, Dinnington, UK).

Statistical analyses

Data were frequently not normally distributed. We used the Mann–Whitney test or Student's *t*-test with Welch's correction to study differences between groups and Spearman correlation for relationships between variables. Chi-square analysis was employed for categorical data. All analyses were performed by use of Prism 5.0a (GraphPad Software, La Jolla, CA, USA) or

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and P-values < 0.05 considered statistically significant.

Results

Clinical and histopathological features

At the time of biopsy, all 18 FS− patients were positive for either anti-Ro/SSA or anti-La/SSB, in comparison with 44/93 of the FS+ patients ($P < 0.001$). Among the FS− patients 16/18 were Ro/SSA+, 6/12 were La/SSB+, and 4 patients were positive for both Ro/SSA and La/SSB. Although the number of patients with unstimulated whole saliva $\leqslant 1.5$ ml/15 min was 51/88 in the FS+ patients compared with 9/18 in the FS− patients, the mean level of unstimulated whole saliva was lower in the FS− patients (1.68 ± 0.22 ml/15 min) compared with the FS+ patients (2.03 ± 0.25 ml/15 min) (Table 1). GC+ patients had the lowest mean salivary secretion (0.95 ± 0.23 ml/15 min) compared with both GC− ($P < 0.01$) and FS− patients ($P < 0.05$).

Minor salivary gland tissue from the seropositive, FS− patients was characterized by diffuse chronic inflammation (Fig. 1D), chronic inflammation and atrophy of glandular tissue/acinar epithelium (Fig. 1E) and fatty cell degeneration (Fig. 1F). In the remaining cases, minor salivary gland tissue had normal morphology (Fig. 1G). Indeed, chronic inflammation presented as focal infiltrates in 8/18 patients, but infiltrates were sparse and FS consequently < 1 (Fig. 1H).

Additional degenerative changes such as interstitial and/or periductal fibrosis were observed in 13/18 biopsies (Fig. 1I). Varying degrees of fibrosis coincided with atrophic minor salivary gland tissue, chronic inflammation, fatty cell degeneration and even non-inflamed salivary gland tissue. In 10/13 patients with interstitial/periductal fibrosis, varying degrees of fatty cell changes were also observed.

Autoantibodies against Ro-52, Ro-60 and La-48

Patient sera were re-evaluated and a trend towards higher levels of all three autoantibodies was observed in FS+ compared with in FS− patients. A similar trend was observed between GC+ and GC− patients. Findings were confirmed by an in-house ELISA [11] (data not shown), though neither test showed significant differences.

Cytokine levels in patient subgroups

Cytokines and focal inflammation. Regarding the presence/absence of focal inflammation, none of the 25 measured cytokines or chemokines proved significantly different when comparing the FS+ and FS− groups. A slight trend towards higher levels of certain cytokines was observed in the FS+ patients, corresponding to increased levels found in GC+ patients (summarized in Table 2). Slight differences in cytokine levels were observed between the GC− and FS−, but in most cases the protein levels were comparable.

Thirteen of 18 FS− patients had clear signs of fibrosis in the minor salivary glands, but no apparent differences could be found when comparing the cytokine levels to the five patients without fibrosis. However, a slight increase in IL-17 levels was observed in patients with fibrosis in the salivary glands compared with those without such changes ($P = 0.097$).

Cytokines and GC-like structures. GC+ patients had significantly increased levels of cytokines IL-1RA, IL-15, IL-17 and INF-α, and of chemokines macrophage inflammatory proteins (MIPs)-1α, MIP-1β, eotaxin and MCP-1 compared with GC− patients, and of IL-1RA and MIG compared with FS− patients. For the remaining cytokines, a trend towards higher protein levels was observed in GC+ patients. Comparing cytokine levels in sera from GC+ and GC− patients revealed a general trend towards higher cytokine levels in GC+ patients and significantly higher levels in 11 of the 25 cytokines investigated (Table 2).

TABLE 2. Comparison of selected cytokine levels in patients with pSS with FS $\geqslant 1$ (FS+) and FS < 1 (FS−) and in individuals with GC formations (GC+) and without GC formation (GC−)

			FS+	
Cytokines (pg/ml)	FS− ($n=18$)	FS+ ($n=97$)	GC− ($n=70$)	GC+ ($n=27$)
IL-1β	120 ± 50	142 ± 24	104 ± 21*	241 ± 62
IL-1RA	3232 ± 1510	4529 ± 989	2772 ± 610*	9084 ± 3050
IL-2R	807 ± 238	1163 ± 197	945 ± 195	1727 ± 486
IL-4	145 ± 13	152 ± 12	132 ± 8*	204 ± 37
IL-12p40	343 ± 79	510 ± 63	392 ± 40*	814 ± 190
IL-15	273 ± 139	323 ± 92	172 ± 46*	715 ± 301
IL-17	76 ± 18	83 ± 13	59 ± 6*	147 ± 44
IFN-α	90 ± 29	106 ± 20	70 ± 11*	201 ± 61
GM-CSF	189 ± 69	286 ± 58	260 ± 68	356 ± 113
MIP-1α (CCL-3)	148 ± 40	162 ± 25	123 ± 16*	266 ± 76
MIP-1β (CCL-4)	436 ± 171	320 ± 52	234 ± 37*	543 ± 153
MIG (CXCL-9)	121 ± 43	182 ± 29	137 ± 22*	299 ± 85
Eotaxin (CCL-11)	156 ± 17	169 ± 10	152 ± 9*	212 ± 26
MCP-1 (CCL-2)	1171 ± 179	1130 ± 81	990 ± 67*	1492 ± 222

Data presented as mean ± S.E.M. *A significant ($P < 0.01$) difference comparing the GC+ and the GC− groups. CCL: CC chemokine ligand.

Cytokines and age. It has been postulated that disease presentation of pSS differs somewhat in early-onset patients compared with those with later onset of disease [12]. When considering the time of biopsy as indicator of disease onset, GC+ patients proved to be significantly younger than the GC− patients. We compared the cytokine profile in patients aged $\leqslant 40$ years and > 40 years, and $\leqslant 50$ and > 50 years in the GC+ and in GC− subgroups. No differences could be detected between the GC+ $\leqslant 40$ years ($n = 9$) and > 40 years ($n = 18$) groups, but IL-17 levels were significantly increased in patients $\leqslant 50$ years ($n = 13$) compared with > 50 years ($n = 14$). In the GC− subgroup, a trend towards higher levels of IL-1RA was observed in the > 40 ($n = 59$) patients ($P = 0.0785$), whereas the $\leqslant 50$ years ($n = 27$) group presented with significantly higher levels of IL-1RA, IL-7, IL-12p40, GM-CSF and MIP-1α compared with > 50 years patients ($n = 43$).

Considering the cohort regardless of GC or FS status ($n = 141$), the picture was similar and no clear differences were found between the $\leqslant 40$ years ($n = 30$) and > 40 years ($n = 111$) subgroups. For the $\leqslant 50$ years ($n = 63$) and > 50 years ($n = 78$) subgroups, a trend towards higher cytokine concentrations in the $\leqslant 50$ subgroup was observed, with significantly increased levels of IL-1β, IL-17, GM-CSF, MIP-1β, MIG and MCP-1, and IL-1RA, IL-12p40, IL-15, IFN-α and MIP-1α. Similar results were found for our sample consisting of 115 FS+ and FS− patients when subdivided accordingly ($n = 51$ for $\leqslant 50$ years and $n = 64$ for > 50 years).

Discussion

Levels of circulating cytokines and chemokines need further exploration in well-characterized patient cohorts. Our results indicate significant variations within cohorts of patients with pSS, also regarding autoantibodies. The FS− patient subgroup was characterized by a high frequency of both Ro/SSA and La/SSB autoantibodies, confirming earlier diagnostic findings in 17/18 patients. This, however, is a consequence of the inclusion criteria used. Autoantibody levels are considered quite stable over time, but fluctuations have previously been observed in SS [13]. Although there was a clear tendency towards higher autoantibody levels in GC+ patients, no significant differences in serum Ro/La autoantibody levels could be detected between the GC+ and GC− subgroups.

Higher local expression of B- and T-cell-attracting chemokines and local autoantibody production in inflamed minor salivary glands in pSS patients has previously been described [6, 14]. In our study, GC+ patients were found to have higher levels of circulating cytokines and chemokines than patients lacking such organisation. Elevated levels of the chemokines MCP-1, MIP-1α, MIP-1β, MIG and eotaxin as seen in the GC+ patients indicate enhanced migration of immune cells. These chemokines are fully capable of attracting B and T cells, macrophages and dendritic cells, all important cell types found in GC, and may play a role in the increased recruitment to and retention of lymphocytes in the salivary glands and the ectopic GC.

A 2-fold increase in IL-1β, a 3-fold increase in IL-1RA and a 4-fold increase in IL-15 levels were observed in GC+ compared with GC− patients. IL-1β is a pro-inflammatory cytokine enhancing T-cell activation, excreted mainly by monocytes and other antigen-presenting cells (APCs), whereas IL-1RA is a receptor antagonist regulating the intensity of IL-1-induced inflammatory responses. IL-15 is a potential pro-inflammatory cytokine [15] produced by monocytes and dendritic cells; it is also a T-cell growth factor, inducing migration of T cells and proliferation of $CD8^+$ memory T cells [16]. Elevated levels of these cytokines may explain the retention of T cells related to GC-like structures and suggest higher T-cell activity in GC+ patients compared with GC− patients.

IFN-α was 3-fold increased in GC+ patients, and is known to up-regulate B cell-activating factor of the TNF family (BAFF) and a proliferation inducing ligand (APRIL) production, thereby inducing B-cell differentiation, and to induce and maintain Th1 cells through dendritic cells and up-regulation of IFN-γ [17]. Focal mononuclear cell infiltrates in salivary glands of patients with pSS include IFN-α-expressing cells [18] and gene expression profiling of minor salivary glands from patients with pSS found several type I IFN-regulated genes to be up-regulated compared with those in healthy controls [19].

The 2.5-fold increase in IL-17 indicates enhanced activity of Th17 cells in GC+ compared with the GC− patients, supported by a 2-fold increase of the IL-23 subunit IL-12p40. IL-17 is involved in the pathogenesis of various autoimmune diseases, and expression of IL-17 has been detected in sera from patients with RA and SLE [20]. Together with IL-1β, IL-23 induces differentiation of Th17 cells, to promote survival and population expansion of Th17 cells [21]. Th17- and IL-23-producing cells have recently been described in salivary glands of both patients and an experimental model for SS [10]. Systemic levels of cytokines connected with Th17 cells differed between subgroups of pSS patients. Whether ectopic GC formation is a result of IL-17 and IL-23 activity, or elevated levels of these cytokines are a consequence of GC formation, remains to be elucidated.

IL-4 is characteristic for the Th2 subset of T cells, involved in activation, proliferation and differentiation of B cells, and antibody class switch to IgE [22]. Studies on KO NOD.B10-$H2^b$ mice showed that a dysfunctional *Il4* gene did not prevent formation of focal mononuclear infiltrates in salivary glands [23], though there was no decrease in salivary flow rates. However, following adoptive transfer of IL-4-secreting Th2 cells, the salivary flow rate was significantly decreased. Interestingly, GC+ patients have significantly reduced unstimulated whole saliva and significantly increased IL-4 levels in serum compared with the GC− and FS− subgroups.

Although SS is far more prevalent in females, the clinical manifestations are not influenced by age or sex [1]. Differences in cytokine levels were not detected when comparing patients $>$ and $<$40 years of age. A slight increase of several cytokines and chemokines was observed when comparing patients $<$ and $>$50 years of age, possibly due to a different disease drive in younger patients compared with middle-aged or elderly patients [1]. Symptoms of SS commonly appear in the fifth decade of life. Previous studies indicate that patients <45 years have a significantly higher frequency of autoantibodies compared with older patients [12]. Thus, one may hypothesize that a change in disease drive occurs between the age of 40 and 50 years, influenced by the changes in female hormone status. Data regarding disease duration were unavailable, but it is tempting to speculate whether the $\leqslant$50 years subgroup represents patients with a more active disease. A trend of longer disease duration was observed in patients with GC compared with those with aggregates [8].

In conclusion, we demonstrate pair-wise variations in cytokine levels between subgroups of patients with pSS. Such differences may be utilized when sub-grouping patients into disease-developing stages and may consequently have implications for diagnosis and treatment regimes.

Rheumatology key messages

- There is a connection between morphological changes in salivary gland inflammation and serum cytokine levels.
- Inflammatory and degenerative changes are found in salivary glands of patients with pSS and not fulfilling FS.

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