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Paper I

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Cellular Basis of Ectopic Germinal Center Formation and Autoantibody Production in the Target Organ of Patients With Sjögren's Syndrome

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Objective. **To investigate functional properties of the germinal center (GC)–like structures observed in salivary glands of patients with Sjögren's syndrome (SS) and to determine the frequency with which such structures develop.**

Methods. **Hematoxylin and eosin–stained sections from 165 minor salivary gland biopsy samples were screened for GC-like structures. Expression of markers for GCs (CD3, CD20, Ki-67, CD35, CD31), adhesion molecules (intercellular adhesion molecule 1, lymphocyte function–associated antigen 1, vascular cell adhesion molecule 1, very late activation antigen 4), chemokines (CXCL13, CCL21, CXCL12), and production of autoantibodies (anti-Ro/SSA and anti-La/SSB) was investigated by immunohistochemistry. Apoptosis was investigated by TUNEL staining.**

Results. **GC-like structures were observed in 28 of 165 patients (17%). When GCs were defined as T and B cell aggregates with proliferating cells with a network of follicular dendritic cells and activated endothelial cells, such microenvironments were found in all patients in whom structures with GC-like morphology were observed. The defined microenvironments were not found in patients without apparent GC-like structures. The GCs formed within the target tissue showed functional features with production of autoantibodies (anti-Ro/SSA and anti-La/SSB) and apoptotic events (by TUNEL staining), and the local production of anti-Ro/SSA and anti-La/SSB autoantibodies was significantly increased (*P* = 0.04) in patients with GC development.**

Conclusion. **Lymphoid neogenesis and functional ectopic GC formation take place in salivary glands of a subset of patients with SS. Our data suggest that the ectopic secondary lymphoid follicles contain all elements needed for driving the autoimmune response. Our findings underscore a key role for the target organ in recruitment of inflammatory cells and propagation of the disease process.**

The chronic autoimmune disorder Sjögren's syndrome (SS) affects exocrine organs, particularly salivary and lachrymal glands (1). Destruction and functional derangement of these glands lead to oral and ocular dryness, in addition to which patients may experience arthralgias and chronic fatigue. B cells and autoantibodies play an important role in the disease, and disturbances in the peripheral and glandular B cell population in patients with SS have been demonstrated (2,3), specifically in terms of clonal expansion (4), changed V- and J-gene segment usage (5), increased plasma cell occur-

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rence in the glands (6), and hypergammaglobulinemia. Autoreactive specificities commonly detected within this antibody pool are the anti-Ro/SSA and anti-La/SSB antibodies, directed against intracellular ribonucleoprotein targets (7).

The glandular lymphocytic infiltration is a progressive feature (8), which, when extensive, may replace large portions of the organs. The glandular infiltrates in some patients closely resemble organized lymphoid tissue with germinal centers (GCs) (9–13). Signs of ectopic lymphoid tissue and lymphoid neogenesis have also been observed in affected tissue in other autoimmune conditions, including synovia in rheumatoid arthritis (14,15), the thymus in myasthenia gravis (16), and the thyroid in Hashimoto thyroiditis (17). In addition, structures resembling GCs can be observed in chronic inflammation without apparent autoimmune origin, as during infection with *Helicobacter pylori* of the gastric mucosa (18) and chronic inflammatory diseases of the liver (19).

Lymphoid neogenesis with formation of ectopic lymphoid follicles in chronic inflammatory disease is a complex process regulated by expression of an array of cytokines, chemokines, and adhesion molecules (for review, see refs. 20 and 21). Several studies have characterized such ectopic lymphoid tissue and shown that it has morphologic features of secondary lymphoid organs with the presence of activated postcapillary high endothelial venules (HEVs), GCs in the interface of B and T cell areas, and expression of adhesion molecules and chemokines mediating homing of naive cells (22,23).

The signals governing normal lymphoid organogenesis during development seem to be involved also in formation of ectopic lymphoid tissue at sites of chronic inflammation (for review, see ref. 24). In particular, the B cell–attracting chemokine CXCL13, required for normal polarization of GCs, has been implicated as a key regulator of lymphoid neogenesis. A number of studies have shown this molecule to be expressed in the glandular infiltrates of patients with SS (11–13). Other chemokines implicated in lymphoid neogenesis are the homing chemokines CCL21 and CXCL12. CCL21 is highly expressed in HEVs and in the T cell area in secondary lymphoid organs and is a chemoattractant for T cells and dendritic cells, while CXCL12 is a potent chemoattractant for both naive and memory T cells and is essential for the earliest stages of B cell lymphopoiesis (for review, see ref. 25). Further, both CCL21 and CXCL12 induce integrin-mediated adhesion of naive cells (26), and CCL21 was recently reported to be ectopically induced in association with chronic inflammatory liver disease (19).

Adhesion molecules are of critical importance in cell trafficking to the inflamed organ. In an animal model of SS, both the adhesion pathways of integrins lymphocyte function–associated antigen 1 (LFA-1; $\alpha 1\beta 2$) and very late activation antigen 4 (VLA-4; $\alpha 4\beta 1$) and their ligands intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) have been shown to be important for lymphocyte migration from blood to inflamed glands (27).

Little is yet known about the functional activity of the ectopic lymphoid tissue seen in autoimmune diseases such as SS. Also, the frequency with which SS patients develop such structures has not been determined. To investigate these issues, salivary gland biopsy samples from patients with focal sialadenitis indicating SS were screened for occurrence of GC-like structures, and 17% of these were found to be positive. High levels of autoantibody production and apoptosis were detected in patients with GCs, indicating a higher level of activity and organization in the glands of these patients. In contrast to previous findings, however, only moderate differences in chemokine and adhesion molecule expression were observed between patients with and those without ectopic GCs. Our data suggest that ectopic GC formation and lymphoid neogenesis take place in the salivary glands of patients with SS. This active participation in the chronic inflammatory process might sustain the autoimmune response and contribute to tissue destruction and loss of function in the exocrine organs of these patients.

PATIENTS AND METHODS

Patients. Three to six minor salivary glands obtained as biopsy samples from patients under investigation for SS (mainly at the Departments of Rheumatology, Otolaryngology, and Head and Neck Surgery at Haukeland University Hospital in Bergen, Norway) were divided. One part was formalin-fixed, embedded in paraffin, and used for routine diagnostics, and the other was snap-frozen and stored at –70°C. In this study, hematoxylin and eosin–stained salivary gland tissue sections from 165 patients evaluated at the Department of Oral Pathology at Haukeland University Hospital during 1991–2002 were screened for GC-like structures. Patients with SS were classified according to the revised European criteria (28). Primary SS was diagnosed in 91 patients and secondary SS in 50 patients. Records were not available or sufficient to classify the remaining 24 patients (focus score $\geq$1; determined as the number of infiltrates with >50 mononuclear cells per 4 mm^2) as having either primary or secondary SS. Additional diagnoses in patients with secondary SS fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for rheumatoid arthritis (29) and systemic lupus erythematosus (30), as well as criteria for

Table 1. Clinical information on patients and subjects included in the study*

	No. of patients or subjects				
	Total	Primary SS	Secondary SS	Age, mean years	Focus score, mean†
SS patients					
Germinal center–positive	13	10	3	50	4.2
Germinal center–negative	6	4	2	62	2.0
Sicca symptom control subjects	5	–	–	49	0

* SS = Sjögren's syndrome.
† Classification criterion for SS, determined as the number of infiltrates with >50 infiltrating lymphocytes per 4 mm^2.

polymyositis (31) and for CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias), chronic active hepatitis, and primary biliary cirrhosis (32).

Among the 165 screened patients, 28 (17%) were found to have infiltrates with structures having GC-like morphology in the formalin-fixed tissue. In total, there was enough snap-frozen tissue to perform all analyses in 13 patients with GCs (Table 1). Six patients diagnosed as having SS, with focus scores ranging from 1 to 4 and lacking GC-like structures in their formalin-fixed biopsy samples, were randomly selected and served for comparison. Biopsy samples from 5 individuals who were evaluated for sicca symptoms, but who did not fulfill the criteria for SS (since they had normal salivary flow and a negative biopsy result), were used as controls. The study was approved by the Committee of Ethics at the University of Bergen (145/96-44.96).

Primary antibodies. The following primary antibodies were used in immunohistochemistry: CD3 (clone T3-4B5, mouse IgG1; Dako, Glostrup, Denmark), CD20 (clone H1[FB1], mouse IgG2a; PharMingen, San Diego, CA), Ki-67 (clone 35, mouse IgG1; Transduction Laboratories, Lexington, KY), CD35 (clone Ber-MAC-DRC, mouse IgG1; Dako), CD31 (clone JC/70A, mouse IgG1; Dako), B cell–attracting chemokine 1/CXCL13 (clone 53610, mouse IgG1; R&D Systems, Minneapolis, MN), secondary lymphoid tissue chemokine (SLC)/CCL21 (goat polyclonal; R&D Systems), stromal cell–derived factor 1/CXCL12 (clone 79014.111, mouse IgG1; R&D Systems), ICAM-1/CD54 (clone 6.5B5, mouse IgG1; Dako), LFA-1/CD11a (clone MHM24, mouse IgG1; Dako), VCAM-1 (clone 1.4C3, mouse IgG1; Dako), and VLA-4 (clone 2B4, mouse IgG1; R&D Systems). Isotype-matched IgG1 and IgG2a antibodies from Dako were used as controls.

Immunohistochemical analyses. Frozen minor salivary glands were sectioned with a cryostat (5-μm sections) and stored at –70°C for a maximum of 3 weeks before analysis. The tissue was fixed at 4°C in 50% acetone for 30 seconds followed by 5 minutes of incubation in 100% acetone. Remaining steps were performed at room temperature. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in Tris buffered saline (TBS; pH 7.6) for 5 minutes. Sections were incubated with primary antibody diluted in TBS for 60 minutes and thereafter with horseradish peroxidase (HRP)–conjugated EnVision+ polymer-HRP antibody (EnVision+ System; Dako) for 30 minutes. Diaminobenzidine (DAB) was used as chromogen. Sections were counterstained with Harris' hematoxylin and mounted before analysis. Between each step, sections were rinsed and washed with TBS twice for 5 minutes each.

For detection of SLC/CCL21, peroxidase blocking was followed by incubation with 10% normal rabbit serum for 20 minutes. Endogenous avidin and biotin were blocked using an avidin–biotin blocking kit (Vector, Burlingame, CA). After incubation for 60 minutes with SLC antibody in TBS, biotinylated rabbit anti-goat antibody (Dako) diluted 1:600 in TBS was added for 45 minutes. Thereafter, sections were incubated with avidin and biotin–labeled HRP (Dako) for 30 minutes. For development, sections were incubated with DAB, followed by counterstaining and mounting as above. Sections were washed with TBS twice for 5 minutes each between each step.

Immunostaining protocol with biotinylated antigens. Recombinant Ro 52-kd, Ro 60-kd, and La proteins were expressed from the pMAL vector (New England Biolabs, Beverly, MA) and purified as previously described (33). Biotinylation of proteins and immunostaining were performed as previously described (2). The recombinant fusion partner maltose binding protein (MaBP) was used in parallel. In short, endogenous HRP, avidin, and biotin activity were blocked as described above, and nonspecific protein binding was blocked by 5% powdered milk and 4% bovine serum albumin (BSA) in TBS for 15 minutes. Sections were then incubated with biotin-labeled Ro 52-kd protein, Ro 60-kd protein, La protein, or MaBP for 45 minutes. Avidin and biotin–labeled HRP (Dako) was used as secondary reagent and incubated with sections for 60 minutes. For development, 3-amino-9-ethyl-carbazole (AEC) was added to the sections for 15 minutes. Sections were counterstained with Harris' hematoxylin. After each step, except for the blocking step, the slides were rinsed and washed in TBS twice for 5 minutes each.

Detection of apoptotic cells by in situ labeling using the TUNEL method. DNA fragmentation during apoptosis was detected in situ by the TUNEL method as previously described (34). Sections (5 μm) were dried and fixed with 10% paraformaldehyde/phosphate buffered saline (PBS) for 30 minutes. Endogenous peroxidase was quenched by exposure to 0.1% H_2O_2 in TBS for 10 minutes. Sections were equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer (0.5*M* cacodylate [pH 6.8], 1 m*M* $CoCl_2$, 0.5*M* dithiothreitol, 0.05% BSA, 0.15*M* NaCl) at 37°C and thereafter covered with TdT buffer containing 0.1 units/μl TdT (Boehringer, Mannheim,

Germany) and 8 nmoles/ml digoxigenin-conjugated dUTP (Boehringer) in a humidified chamber for 60 minutes at 37°C. The reaction was stopped by washing in TBS supplemented with 5% fetal calf serum (FCS) followed by incubation with sheep anti-digoxigenin IgG (Boehringer) diluted to 5 μg/ml in blocking solution for 60 minutes. After soaking in 10% pooled human sera, 5% rabbit sera, and 2% FCS in TBS, sections were incubated with HRP-conjugated rabbit anti-sheep antibodies (Dako) diluted 1:100 in blocking solution for 60 minutes.

TUNEL-positive cells were visualized by incubation with AEC dissolved in DMSO (10 mg/ml) with 0.3% H_2O_2 for 15 minutes, counterstained in Harris' hematoxylin, and mounted. Sections were washed in TBS between each step, and all steps were carried out at room temperature unless indicated otherwise. Negative control tissue sections from each patient were incubated as described above, omitting dUTP.

Evaluation of staining. Between 3 and 6 minor salivary glands from each patient were evaluated in all stainings performed. Cells were counted using a light microscope with a grid and a 40× objective. For analysis of adhesion molecule expression, TUNEL-positive cells, and autoantibody-producing cells, an average of 5–6 focal infiltrates (~400 focal infiltrating mononuclear cells) were analyzed within randomly selected fields across the whole section. In specimens with fewer than 5 infiltrates, more than one area of infiltrating mononuclear cells within the same focal infiltrate was counted. Cells with >50% of the cell membrane immunohistochemically stained for ICAM-1, LFA-1, VCAM-1, and VLA-4 were quantified as positive. In quantifying TUNEL reactivity, only stained nucleus or nuclear fragments that morphologically matched apoptotic cells were counted as positive. The investigated chemokines were mainly expressed by epithelial cells. Positive acini and ductal epithelial cells in each glandular section were counted and expressed as the mean of 3–6 glands.

Enzyme-linked immunosorbent assay (ELISA) for detection of antibodies against Ro 52-kd, Ro 60-kd, and La proteins. ELISA was performed as previously described (33) with minor modifications. In short, medium-binding 96-well plates (Nunc, Odense, Denmark) were coated (1 μg/well) with recombinant full-length Ro 52-kd protein, Ro 60-kd protein, La protein, or wild-type vector MaBP diluted in carbonate buffer (pH 9.6). Plates were blocked with PBS–0.05% Tween (PBST)/5% milk powder, and sera were tested at serial dilutions from 1:500 to 1:1,000,000 in PBST/1% milk powder. Bound antibodies were detected by affinity-purified alkaline phosphatase–conjugated anti-human IgG antibodies (Dakopatts, Glostrup, Denmark). Phosphatase substrate tablets (Sigma, St. Louis, MO) dissolved in diethanolamine buffer (pH 9.6) were used as substrate. The absorbance was measured at 405 nm. All steps were performed at room temperature except coating, which was performed at 4°C. A standard curve was calculated from a previously identified high-titer Ro and La–positive serum run in parallel each time, and reactivity was expressed as units.

Statistical analysis. The Mann-Whitney test was used for statistical analysis of nonparametric data. The Spearman correlation coefficient was calculated for testing correlation of parameters. Chi-square analysis was employed for comparing GC occurrence in primary and secondary SS. *P* values less than 0.05 were considered statistically significant.

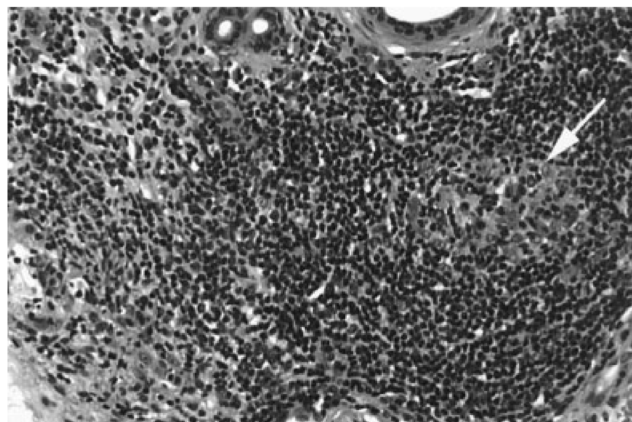

Figure 1. Germinal center (GC)–like structure in a hematoxylin and eosin–stained, formalin-fixed minor salivary gland section from a patient with Sjögren's syndrome. **Arrow** denotes proposed GC dark zone consistent with proliferating cells, surrounded by densely packed cells in the proposed GC light zone. (Original magnification × 200.)

RESULTS

Formation of ectopic GCs in salivary glands of patients with SS. Formalin-fixed salivary gland biopsy samples from 165 patients with SS were screened for GC-like structures. Among the screened patients, 28 (17%) were found to have infiltrates with structures having GC-like morphology (Figure 1). There was no statistically significant difference between occurrence of these structures in patients with primary SS and in those with secondary SS. In 13 of these patients (10 with primary SS and 3 with secondary SS), frozen tissue was available for performing all stainings. Salivary gland tissue from patients diagnosed as having SS, with a focus score ranging from 1 to 4 but lacking GC-like structures in the formalin-fixed biopsy samples, were randomly selected and used for comparison. Biopsy samples from 5 subjects evaluated for sicca symptoms but not fulfilling the criteria for SS were used as additional controls.

To characterize the GC-like structures, patient biopsy samples were analyzed for a number of cell markers, including markers for T cells (CD3), B cells (CD20), proliferating cells (Ki-67), follicular dendritic cells (FDCs; CD35), and endothelial cells (CD31). Glands with clusters of proliferating lymphoid cells and a network of FDCs in large lymphocytic infiltrates with morphologic features mimicking those of GCs in lymphoid tissue were classified as GC+, while patients

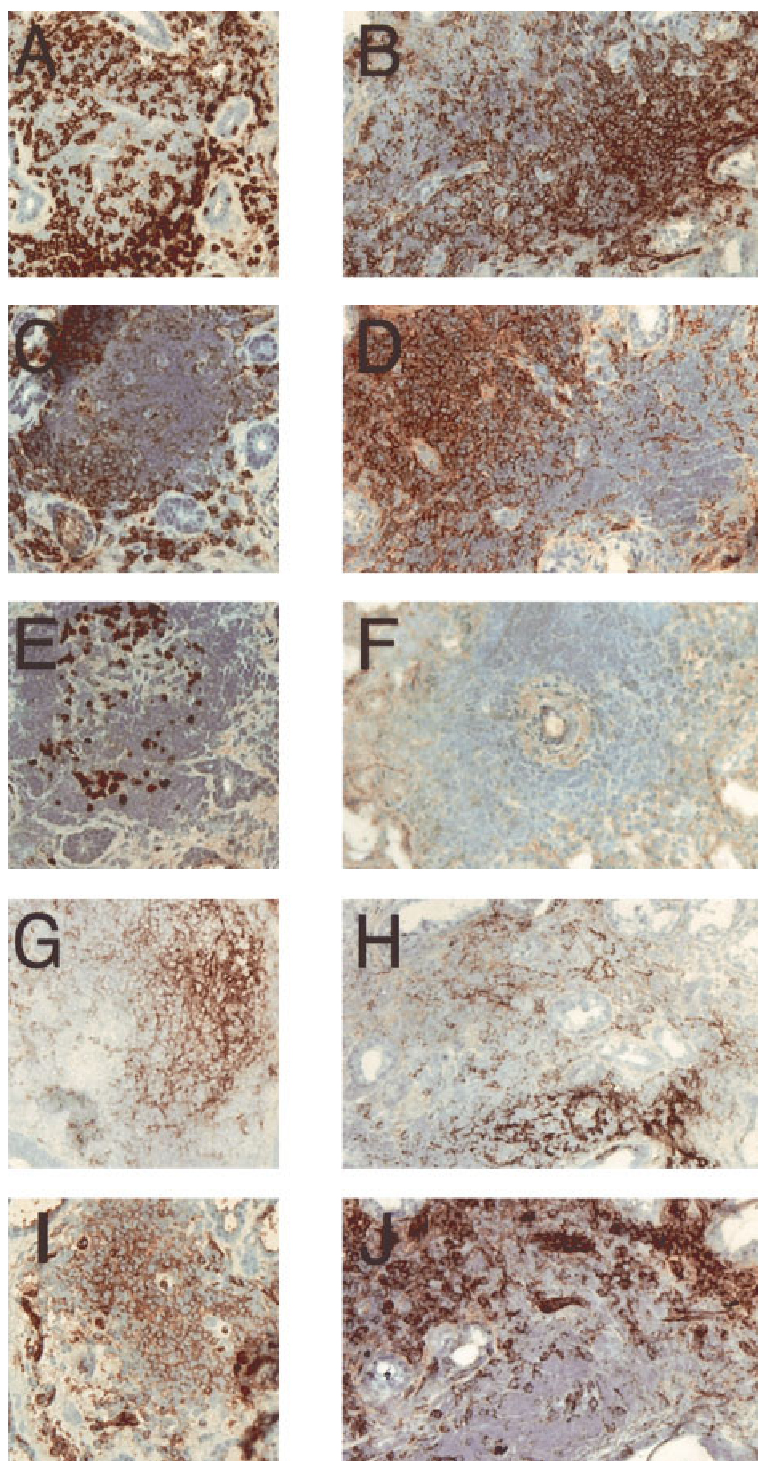

Figure 2. Immunohistochemical staining for cell markers in salivary gland sections from patients with (**A**, **C**, **E**, **G**, and **I**) or without (**B**, **D**, **F**, **H**, and **J**) germinal centers. Sections were stained with antibodies to T cells (anti-CD3) (**A** and **B**), B cells (anti-CD20) (**C** and **D**), proliferating cells (anti–Ki-67) (**E** and **F**), follicular dendritic cells (anti-CD35) (**G** and **H**), and endothelial cells (anti-CD31) (**I** and **J**). (Original magnification × 200.)

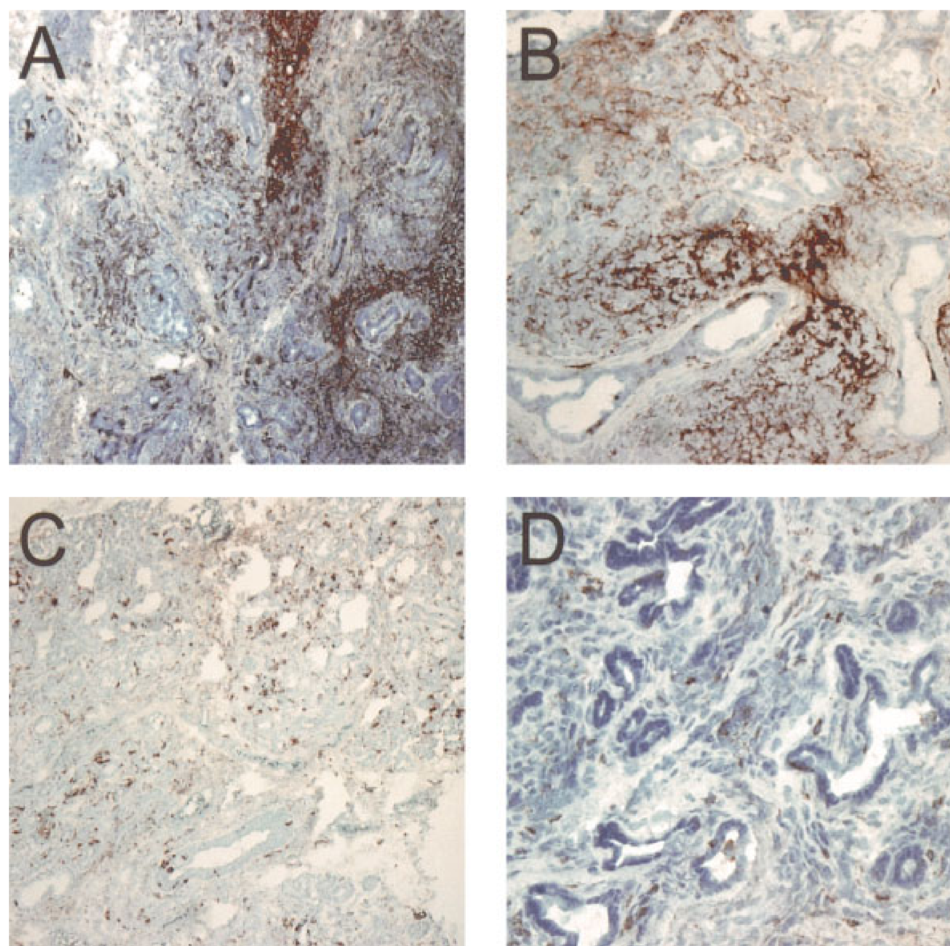

Figure 3. Distribution of follicular dendritic cells (FDCs) in germinal center (GC)+ and GC− salivary glands. Immunohistochemical staining for CD35 was used to visualize FDCs. A network of FDCs could be observed with the T and B cell aggregates in GC-containing salivary glands (two different patients) (**A** and **B**), while the distribution was scattered and no networks were observed in GC− glands (two different patients) (**C** and **D**). (Original magnification × 100 in **A** and **C**; × 200 in **B** and **D**.)

lacking one of these features were defined as GC− (Figure 2). Most GC− patients lacked proliferating cells or FDC networks, although most displayed a scattered distribution of FDCs (Figure 3). In both groups of patients (GC+ and GC−), the lymphocytic infiltrates were dominated by CD3+ T cells, although aggregates of both CD3+ T cells and CD20+ B cells were found in areas with proliferating cells in the GC+ group. In a few patients lacking GCs (GC−), smaller aggregates of T and B cells could be found in association with a scattered distribution of FDCs, but these infiltrates lacked the marker for proliferating cells (Ki-67). Accordingly, the most distinguishing features between GC+ and GC− infiltrates were the organization of FDCs and the presence of proliferating cells. Blood vessels were identified using a marker for endothelial cells (CD31) and by histologic observations, and were equally distributed in salivary glands both in patients with and in those without GCs.

Local anti-Ro and anti-La autoantibody production and apoptotic events appearing in association with GCs in salivary glands of patients with SS. Using biotinylated recombinant antigens as probes, we investigated local production of the anti–Ro 52, anti–Ro 60,

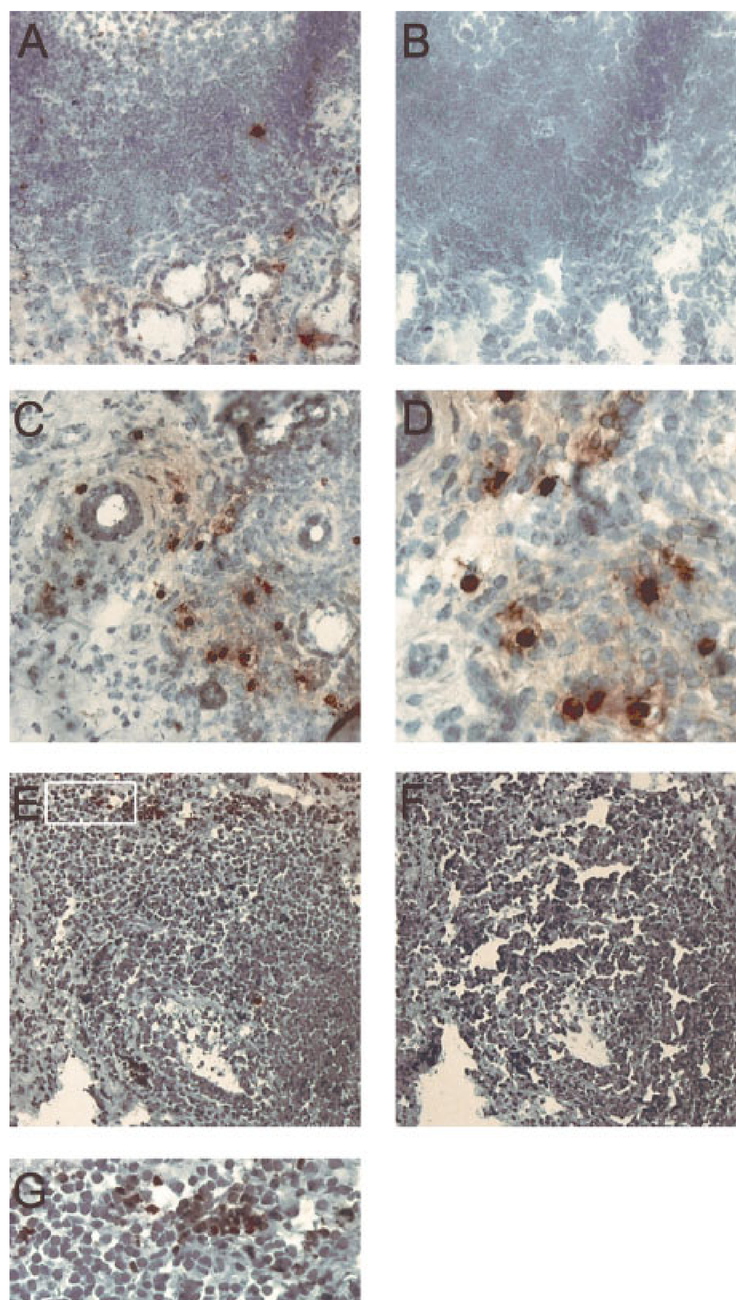

Figure 4. Local production of autoantibodies and apoptotic cells in association with large focal infiltrates in salivary gland sections. Biotinylated recombinant proteins were used for staining and visualization of antibody-producing cells. **A,** Cells producing antibodies to the Ro 52 autoantigen encountered at the margin of large infiltrates and in interstitial tissue. **B,** Negative control staining with the recombinant fusion partner maltose binding protein of the same area shown in **A**. In patients with a high frequency of autoantibody-producing cells, these were not restricted to large infiltrates, but were also observed **C**, in smaller infiltrates. **D,** Higher magnification of same area shown in **C**. The distribution of apoptotic cells coincided with that of autoantibody-producing cells. Apoptotic cells, detected with the TUNEL method, were mainly encountered **E**, at the margin of large infiltrates. **F,** Negative control staining of the same area shown in **E**. **G,** Higher magnification of boxed area shown in **E**. (Original magnification × 200 in **A–C**; × 400 in **D**; × 100 in **E** and **F**; × 300 in **G**.)

Table 2. Summary of data obtained by immunohistochemical staining for chemokines, autoantibody-producing cells, and apoptotic cells in salivary glands*

Patient or control (C) subject†	GC status‡	Focus score§	Chemokines¶			Autoantibody-producing cells#			Apoptosis**
			CXCL13	CCL21	CXCL12	Anti–Ro 52	Anti–Ro 60	Anti-La	
1	Positive	12	+	+	+	+++	++	++	++
2	Positive	2	+	+	+	+	−	−	−
3	Positive	5	+	+	+	+++	+	++	+
4	Positive	4	+	++	++	+++	+	+	+
5	Positive	7	++	++	++	+	++	+	++
6	Positive	4	+	+	+	−	+	−	−
7	Positive	1	+	+	++	−	−	−	++
8	Positive	3	+	+	+	++	+	+	+
9	Positive	1	++	+	++	−	−	−	−
10	Positive	6	+	+	+	+	−	+	−
11	Positive	2	+	+	+	−	−	−	−
12	Positive	5	+	+	+	+	+	++	−
13	Positive	3	+++	+++	++	−	−	−	−
14	Negative	1	+	+	+	−	−	−	−
15	Negative	1	+	++	+	−	−	−	−
16	Negative	4	+	+	+	−	−	−	+
17	Negative	3	++	++	++	−	−	−	−
18	Negative	1	+	+	+	−	++	+	−
19	Negative	2	+	+	+	+	−	−	−
C1	NA	0	−	ND	−	ND	ND	ND	−
C2	NA	0	−	ND	+	ND	ND	ND	−
C3	NA	0	−	ND	+	ND	ND	ND	−
C4	NA	0	−	ND	−	ND	ND	ND	−
C5	NA	0	−	−	−	−	−	−	−

* GC = germinal center; NA = not applicable; ND = not done.
† Control subjects were 5 individuals evaluated for sicca symptoms but not fulfilling the criteria for Sjögren's syndrome (SS).
‡ Positive = GC-like structures with proliferating cells and follicular dendritic cells (FDCs) in network formation (n = 13 patients); negative = infiltrates without proliferative activity or FDC networks (n = 6 patients).
§ Classification criterion for SS, determined as the number of infiltrates with >50 infiltrating lymphocytes per 4 mm^2.
¶ − = negative; + = 1–5 positive acini per salivary gland; ++ = >5 positive acini per salivary gland; +++ = majority of acini with strong positive staining.
# − = negative; + = 1–10 positive cells/mm^2; ++ = 11–20 positive cells/mm^2; +++ = >20 positive cells/mm^2.
** By TUNEL staining: − = <0.01% apoptotic cells in focal infiltrates; + = 0.01–0.02% apoptotic cells in focal infiltrates; ++ = >0.02% apoptotic cells in focal infiltrates.

and anti-La autoantibodies within the salivary gland inflammatory infiltrates (Figure 4). The frequency of anti-Ro–positive and anti-La–positive cells was significantly higher in patients with GC formation than in patients without GCs ($P = 0.04$) (Table 2). Autoantibody-producing cells were mainly localized in the periphery of the inflammatory infiltrates and in the interstitium (Figure 4).

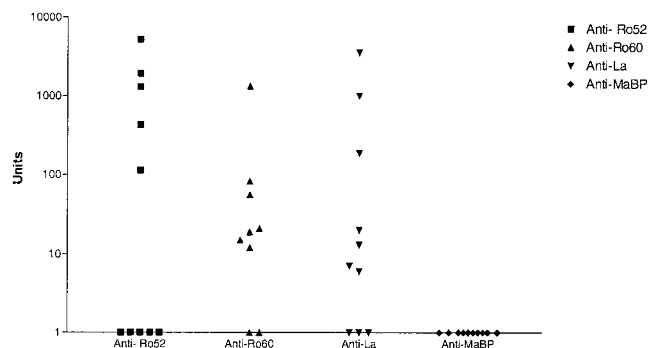

Figure 5. Analysis of serum autoantibody levels by enzyme-linked immunosorbent assay. Recombinant Ro 52, Ro 60, and La antigens, as well as wild-type vector maltose binding protein (MaBP), were used for detection.

Serum levels of the corresponding autoantibodies were analyzed in 10 patients from whom sera were available (Figure 5). A strong correlation was found between local salivary gland production and systemic levels of anti–Ro 52 antibodies ($r = 0.81$, $P = 0.005$) and between local salivary gland production and systemic levels of anti-La antibodies ($r = 0.75$, $P = 0.01$). The same tendency was noted for anti–Ro 60 antibodies, but the correlation did not reach statistical significance ($P > 0.05$).

Apoptosis was a relatively rare event in the glandular biopsy samples studied. The frequency of apoptotic cells located in the focal infiltrates was <0.02%, but somewhat higher in patients with GC formation compared with patients lacking these struc-

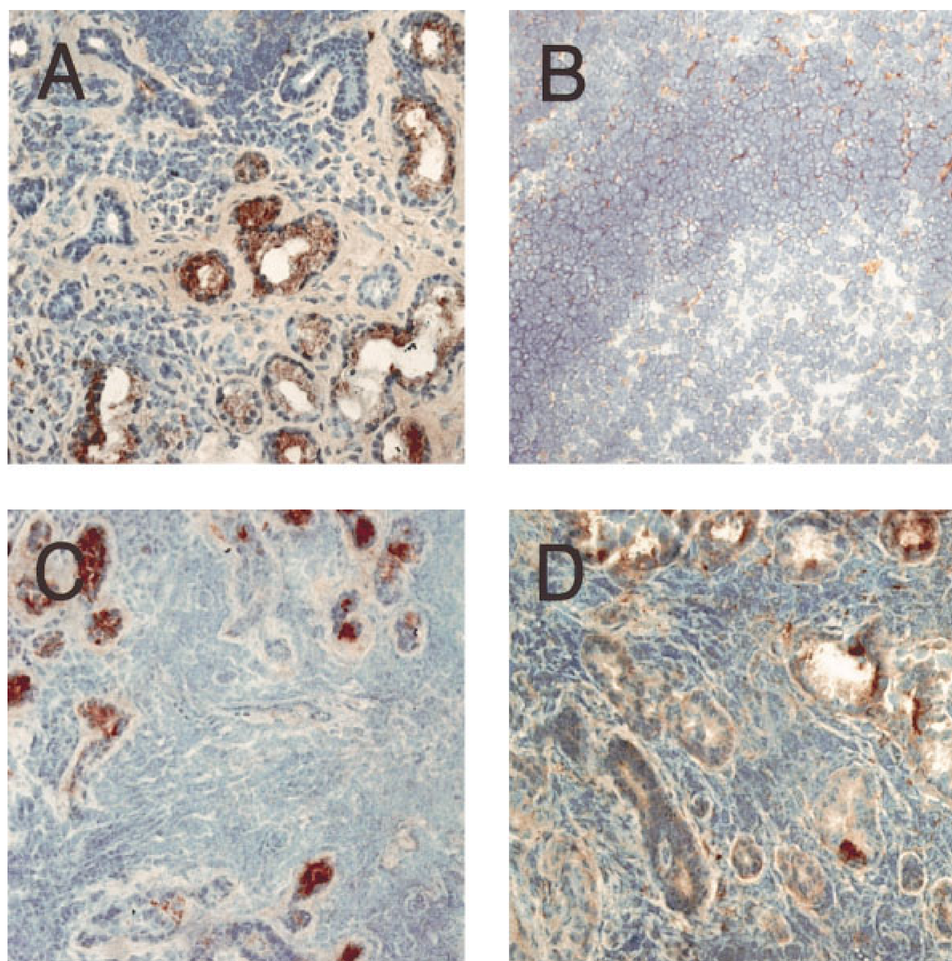

Figure 6. Immunohistochemistry with staining for expression of the chemokines CXCL13, CXCL12, and CCL21 in patients with Sjögren's syndrome. Salivary gland acinar and ductal epithelial cells expressed CXCL13 (**A**), CXCL12 (**C**), and CCL21 (**D**). Positive and negative acini/ducts were observed within the same section. Normal tonsil tissue displayed CXCL13 staining in a reticular pattern in the margin of germinal centers (**B**). (Original magnification × 200.)

tures (Table 2). Although a clear spatial correlation was not observed, the distributions of autoantibody-producing cells and apoptotic cells were similar; both categories of cells were mainly encountered at the margin of large focal infiltrates or interstitially (Figure 4).

Trafficking of lymphoid cells to salivary glands of patients with SS. Based on the central role of the chemokines CXCL13, CCL21, and CXCL12 in secondary lymphoid organ and GC formation, we investigated the expression of these molecules. The selected chemokines were expressed by acinar and ductal epithelial cells in all SS patients investigated (Figure 6), but no correlation was found between degree of expression and presence/absence of GCs in the gland (Table 2). Control subjects did not show any expression of CXCL13 or CCL21, while 2 control subjects showed a weak staining for CXCL12.

Adhesion molecules ICAM-1 (CD54), LFA-1 (CD11a), VCAM-1, and VLA-4 were all highly expressed in large lymphocytic infiltrates (Figure 7). In particular, high expression was noted in close proximity to GCs. In the biopsy samples from SS patients, expression of ICAM-1 was identified on mononuclear cells, both in focal infiltrates and interstitially, as well as on endothelium and fibroblast-like cells (Figure 7). Acinar and ductal epithelial cells were negative, except for the basement membrane–like structures of acini and intercalated ducts in close relation to infiltrates. LFA-1, the

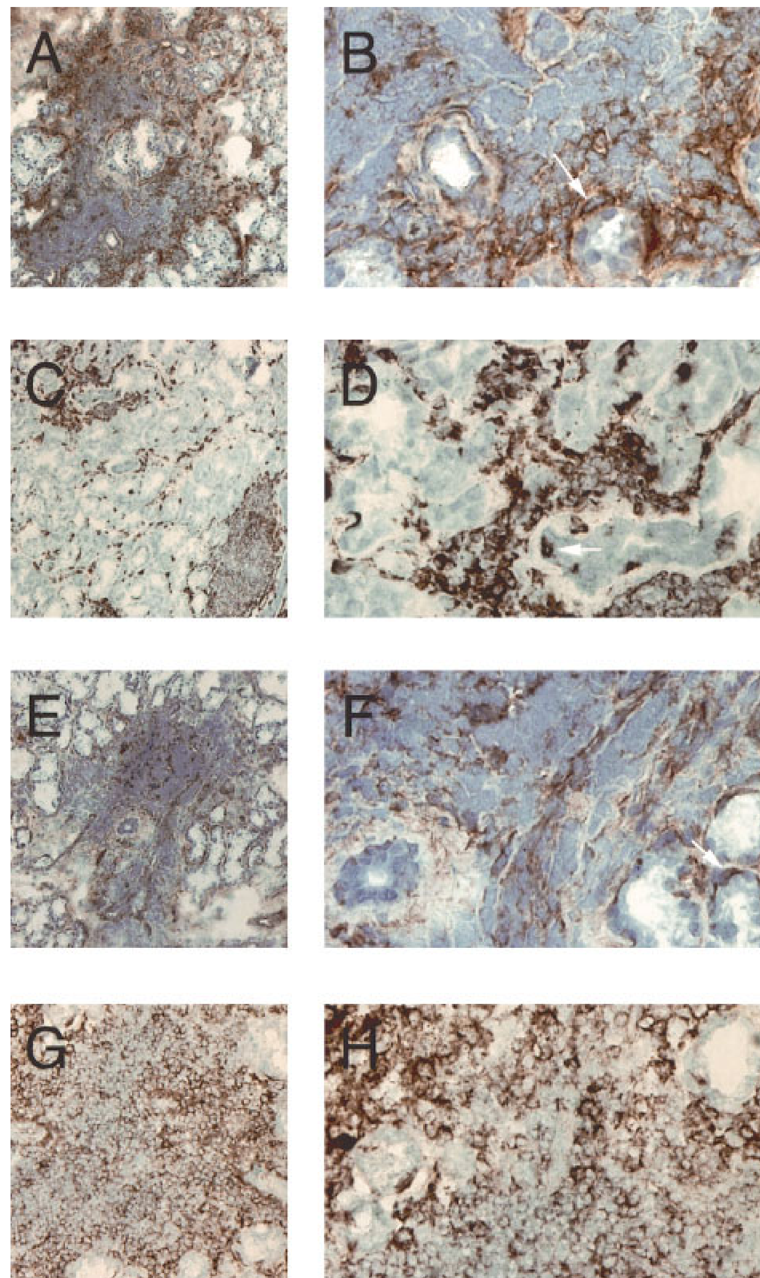

Figure 7. Expression of adhesion molecules in lymphocytic infiltrates in salivary gland sections from patients with Sjögren's syndrome analyzed by immunohistochemistry. **A** and **B**, Intercellular adhesion molecule 1 (ICAM-1; CD54); **C** and **D**, lymphocyte function–associated antigen 1 (LFA-1; CD11a); **E** and **F**, vascular cell adhesion molecule 1 (VCAM-1); **G** and **H**, very late activation antigen 4 (VLA-4). ICAM-1 was expressed on infiltrating mononuclear cells (**A** and **B**), both in focal infiltrates and interstitially, as well as on fibroblast-like cells (**arrow** in **B**). No ICAM-1 expression was detected on acinar or ductal epithelial cells, except for the basement membrane structures of acini/intercalated ducts in close relation to mononuclear cell infiltrates. **C** and **D**, LFA-1, the integrin interacting with ICAM-1, was expressed on mononuclear cells in infiltrates and interepithelially (**arrow** in **D**), both in acini and in ductal epithelium. VCAM-1 was expressed on endothelial cells and on fibroblast-like cells (**arrow** in **F**) and displayed a reticular staining pattern in large infiltrates (**E** and **F**). VLA-4 was expressed on infiltrating mononuclear cells, both in focal infiltrates and interstitially (**G** and **H**). (Original magnification × 100 in **A**, **C**, **E**, and **G**; × 400 in **B**, **D**, **F**, and **H**.)

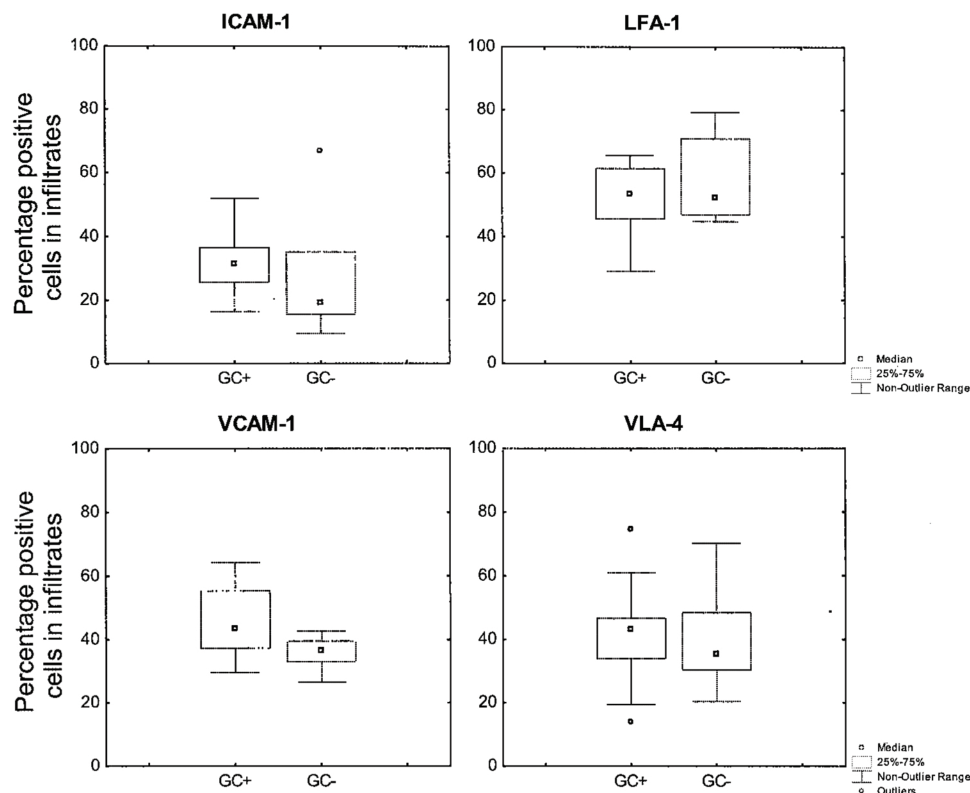

Figure 8. Percentages of cells expressing ICAM-1, LFA-1, VCAM-1, and VLA-4 (of the total number of focal mononuclear cells in a defined area) in patients with or without germinal centers (GC+ or GC−, respectively). A relative up-regulation of ICAM-1 and VCAM-1 was observed in salivary glands of GC+ Sjögren's syndrome patients, while the relationship was reversed for LFA-1. Equal numbers of cells expressing VLA-4 were observed in GC+ and GC− patients. See Figure 7 for other definitions.

ligand of ICAM-1, was expressed on mononuclear cells, in infiltrates, and intercellularly in both acinar and ductal epithelium. Lower expression of ICAM-1 and LFA-1 was observed in the central region of large infiltrates in patients with GCs. VCAM-1 was expressed on endothelial and fibroblast-like cells. Additionally, a reticular staining pattern resembling the distribution of FDCs was observed in infiltrates. However, this was only seen in more extensive infiltrates. VLA-4, the ligand of VCAM-1, was expressed on infiltrating mononuclear cells, both in focal infiltrates and interstitially.

The percentages of cells expressing ICAM-1, LFA-1, VCAM-1, and VLA-4 (of the total number of focal mononuclear cells in the corresponding area) were compared between patients with and those without GC-like structures. Interestingly, a relative increase of ICAM-1 and VCAM-1 in salivary glands of SS patients with GCs (GC+ patients) was observed, while the relationship was reversed for LFA-1. However, these differences did not reach the level of statistical significance. Equal numbers of cells expressing VLA-4 were observed between GC+ and GC− patients (Figure 8).

DISCUSSION

Destruction of salivary glands in patients with SS is related to chronic inflammation, manifested by focal lymphocytic infiltration. By using a series of phenotypic markers, we identified an architecture and activity in the salivary glands of SS patients normally found in organized secondary lymphoid tissue. The observations of large aggregates of B and T cells and dense populations of proliferating cells in close proximity to FDC networks indicate that GCs are formed in patients with SS. High expression of adhesion molecules and chemokines, involved in attracting and organizing lymphocytes in secondary lymphoid organs, as well as apoptosis and local production of autoantibodies against the Ro/SSA and

La/SSB proteins were observed in the salivary glands of SS patients. Taken together, we demonstrate that highly organized structures are formed and activity normally associated with secondary lymphoid organs takes place in the target organ of patients with SS.

GC-like structures could be identified in 17% of the 165 investigated patients. No significant difference in their occurrence was observed between primary and secondary SS. For diagnostic purposes, at least 5 minor salivary glands are recommended. This amount is considered sufficient to provide a representative sample of material for the generalized exocrinopathy, lending support to a fairly proper assessment of GC development in our study. To our knowledge, this is the first report to describe the scanning of a larger sample of material obtained from patients evaluated for SS, although it is possible that the occurrence of GC-like structures might be different in the major glands. The frequency we found in minor salivary glands is nevertheless comparable with the incidence in synovial tissue of patients with rheumatoid arthritis, where GCs were detected in 23% of the biopsy samples (14).

In the minor salivary glands, we observed GCs only in manifest infiltrates, and in most cases proliferating areas were seen in several glands from the same patient. This indicates that a mass of cells is required to provide the microenvironment where GCs may develop, but that when it is initiated, the GC formation is a general phenomenon occurring at multiple sites. Autoantibody-producing cells were detected at significantly higher frequency and in higher numbers in patients with GCs compared with patients lacking such structures, indicating a functional activity in the organized lymphocytic infiltrates. A correlation was also detected between the presence of local antibody-producing cells in the salivary glands and higher anti-Ro and anti-La levels in sera as we previously described (2,35), suggesting a more active disease status in these patients.

Reports of clonal B cell proliferation and hypermutation within the salivary glands of patients with SS (9), together with direct and indirect signs of local production of autoantibodies (13,36,37), support the interpretation of functional activity in the GCs. Differences in the microenvironment and structure, as observed in chronically inflamed tonsils compared with traditional GCs (38), possibly in combination with easy access to autoantigens, might contribute to a disturbance in the selection process allowing autoreactive clones to develop more frequently. Even though local production of autoantibodies in the salivary glands is correlated with higher antibody levels, it should be noted that the autoantibodies are not confined to patients with ectopic GCs, indicating the possibility of anti-Ro/anti-La autoantibody production taking place at additional sites. In this respect, some autoantibody-producing cells were found to recirculate (37), but production elsewhere, including bone marrow and spleen, remains to be investigated.

Hypergammaglobulinemia and persisting levels of autoantigen-specific IgM (39) have been demonstrated in SS patients, and one might speculate that at least some of the plasma cells encountered within the inflamed glands belong to the pool of long-lived plasma cells, for which inflamed tissue has recently been shown to provide survival niches equivalent to those in the bone marrow (40). A regulation of plasma cell survival has been suggested to be mediated by competition for survival niches in the bone marrow; de novo formation of survival niches in chronically inflamed tissue might unbalance this regulation and promote survival of autoreactive B cell clones, thus further sustaining the disease process.

Moderate levels of apoptotic cells were detected both in patients with and in those without GCs. The apoptotic cells were encountered at the margins of large infiltrates, as well as in interstitial space, partly coinciding with the localization of autoantibody-producing cells, in concordance with previously reported findings (2,13,34). The low numbers of apoptotic cells observed could be a result of B cells escaping apoptosis and proper selection in the ectopic GCs. Indeed, attenuated apoptosis of B cell–activating factor–expressing cells has recently been demonstrated in SS (41). The low apoptosis rate might also partly be explained by the fact that apoptosis is a relatively short event, spanning ~3 hours, possibly making detection difficult with the TUNEL method used.

The investigated chemokines CXCL13, CCL21, and CXCL12 were expressed by acinar and ductal epithelial cells in all SS patients studied. Notably, no correlation was observed between level of chemokine expression and presence of GCs or size of lymphocytic infiltrates, implying a constant level of chemokine expression maintained during the entire inflammatory process in the investigated patients. This partly contradicts the interpretation of previous findings, where expression of CXCL13 was suggested to be closely associated with ectopic GC development (11). In another study (14), tissue containing ectopic GC reactions was reported to have a 10–20-fold increase in transcription levels of CXCL13 and CCL21, although it should be

noted that these chemokines were also detected in tissues lacking such structures. In conformity with findings of others, our results suggest that the epithelial cells are active participants in the inflammatory process in the glands of SS patients, both in producing chemokines and cytokines (11,13,42–44) and in expression of major histocompatibility complex class II (45) and costimulatory molecules (46).

The adhesion molecules LFA-1 and VLA-4 were expressed in all salivary glands investigated. Patients with GCs showed a lower expression of LFA-1 in the central region of large infiltrates, possibly indicating a decrease in LFA-1–mediated adhesion interactions when infiltrates are being organized. LFA-1 has a central role in lymphocyte trafficking and interaction, as implied by the findings of in vitro experiments where antibodies against LFA-1 have been reported to inhibit adhesion of both naive and activated T cells (for review, see ref. 47). Patients lacking LFA-1 have abnormalities in a wide spectrum of adherence-dependent functions of granulocytes, monocytes, and lymphoid cells (48).

ICAM-1 was highly expressed on infiltrating mononuclear cells in all biopsy samples investigated. Similar to LFA-1, expression of ICAM-1 was lower in the central region of large infiltrates. ICAM-1 was also detected on fibroblast-like cells, as reported earlier in SS patients (49). In contrast to the findings of Kapsogeorgou and colleagues (49), we could not detect ICAM-1 on the epithelium, except for a discrete staining in the basement membrane region where acini or ducts appeared in close proximity to infiltrates. VCAM-1 was expressed on endothelial and fibroblast-like cells. A reticular staining pattern for VCAM-1 on mononuclear cells was observed in large infiltrates, possibly representing FDCs (10). In concordance with the findings of others, we found increased expression of adhesion molecules in the glands of patients with SS, suggesting activation and active recruitment of inflammatory cells (49–51). The ratios of both ICAM-1– and VCAM-1–expressing cells were increased in focal infiltrates in SS patients with GCs, indicating a higher degree of activation and lymphocyte trafficking in the salivary glands of these patients, although differences were not statistically significant.

An association of GC development with the increased risk of B cell lymphomas seen in patients with SS has been suggested (52), and formation of proliferating GCs has been proposed to contribute to the malignant transformation and development of mucosa-associated lymphoid tissue–type lymphoma (53). Patients with SS and chronic *H pylori* infection are at an elevated risk for developing lymphomas, possibly related to prolonged lymphocytic activation in the target organs of these patients. Disturbances of the B cell maturation have been demonstrated in patients with SS (3,54), part of which might be explained by overstimulation and neglected selection in ectopic GCs in the affected glands. To include assessment of GCs in the clinical routine evaluation of diagnostic minor salivary gland biopsy samples might therefore prove beneficial in predicting lymphoma development, suggesting a need for prospective studies confirming the association. Also, biopsy sample size will be important in obtaining representative material for such a study.

In conclusion, our results suggest that formation of ectopic lymphoid microstructures in nonlymphoid organs participates in the pathogenesis of organ-specific autoimmunity, here illustrated by the chronic inflammatory disease SS. Whether this is a primary or secondary event in the initiation of the inflammatory and autoimmune disease process remains to be investigated further; our observations nonetheless underscore a key role of the target organ in cell recruitment and disease progression. The involvement of salivary glands as a site of ectopic GC formation and of the selection of high-affinity autoantibodies mediating this autoimmune state suggests novel targets for future immunomodulatory therapeutic strategies.

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