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CHARACTERIZATION OF PRELYMPHOMATOUS STAGES OF B CELL LYMPHOPROLIFERATION IN SJÖGREN'S SYNDROME

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Objective. **To determine whether the prelymphomatous stages of B cell lymphoproliferation in Sjögren's syndrome (SS) may be better characterized by the integration of clinical, pathologic, and molecular data, the latter focusing on the expansion, persistence, and dissemination of clonal B cells in the course of the disease.**

Methods. **Multiple tissue lesions (synchronous from different tissues and metachronous from the same tissue) were evaluated in biopsy specimens obtained from 6 consecutive patients with SS who had an associated lymphoproliferative disorder. Fully benign gastric lesions were evaluated in tissue from an additional 11 patients with SS who had no associated lymphoproliferative disorder. Multiple and complementary molecular analyses of B cell clonality were used: Southern blot, polymerase chain reaction, single-strand conformation polymorphism, DNA sequencing, and hybridization with clonospecific oligoprobes. All the patients were then *strictly followed up* for the appearance of lymphoma.**

Results. **Different scenarios of SS-associated B cell lymphoproliferation were identified: 1) the ongoing expansion of the same dominant clone, localized or disseminated, in tissue from 2 patients, 1 of whom later developed an overt B cell lymphoma; 2) different dominant clones in different synchronous or metachronous tissues from the remaining 4 patients with an associated lymphoproliferative disorder; and 3) small oligoclonal expansions in 7 of the 11 benign gastric lymphoid infiltrates.**

Conclusion. **Prelymphomatous B cell lymphoproliferation in SS was better characterized following integration of the findings. The different types of B cell clonal expansion (oligoclonal or monoclonal, smaller or larger in size, fluctuating or established, localized or disseminated) may imply a different risk of lymphoma progression. An accurate clinical, histopathologic, and molecular characterization may therefore be crucial in future studies aimed at clarifying the pathobiology of SS-associated lymphoproliferation.**

B cell clonal expansion is an early event in the course of Sjögren's syndrome (SS), a disease which has been shown to be an important human model of B cell lymphomagenesis (1–5). A full-blown B cell non-Hodgkin's lymphoma (NHL) develops in about 5% of SS patients, but earlier stages of B cell lymphoproliferation are more frequent (3,4,6). In histopathologic analyses of both SS-related and SS-unrelated lymphoproliferation, these stages may range from clearly benign lymphoid infiltrates to lesions that can be difficult to differentiate from low-grade B cell NHLs.

A better characterization of such progression has very important clinical implications and is crucial when investigating the pathobiology of SS-related lymphoproliferation (3,4). In particular, there is no general agreement on lymphoproliferative lesions that have evidence of local B cell clonal expansion and that involve the major salivary glands (the main target of lymphoproliferation in SS). These lesions have been considered to be either benign or early malignant (7–11). The historic

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term, pseudolymphoma, has been frequently used in those cases of SS that develop a clinical picture suggestive of malignancy, but which cannot be clearly classified as malignant, even after tissue biopsy (3). SS-unrelated pseudolymphomas, benign lymphoid hyperplasias (12), and atypical lymphoproliferative disorders (13,14) are also poorly characterized. To address this issue, accurate studies that strictly combine a careful clinical approach with up-to-date technologies are critical.

To better characterize the prelymphomatous stages of B cell lymphoproliferation in SS, the approach followed in the present study included 1) the use of multiple molecular analyses of B cell clonality in a spectrum of SS-associated lesions that were well characterized according to accepted histopathologic criteria and that involved tissues in which NHL may develop (3,4,13–18), and 2) the analysis of multiple biopsy specimens, synchronous from different tissues and metachronous from the same tissue, in the followup of consecutive SS patients who had an associated lymphoproliferative disorder.

Such an approach proved to be useful to highlight different scenarios of prelymphomatous B cell lymphoproliferation in SS, including 1) the ongoing expansion of a single dominant B cell clone in the same lymphoproliferative lesion, with the possible dissemination and local overexpansion of the same clone in different tissues (putative, more advanced stages of disease progression toward B cell malignancy); 2) different dominant clones in different synchronous and metachronous biopsy specimens, indicating a fluctuating rather than an established monoclonal B cell lymphoproliferative disorder (a putative earlier stage, involving a less severe risk of subsequent malignancy); and 3) small oligoclonal B cell expansions in fully benign pathologic lesions from patients without lymphoproliferative disorders, which appeared of minor clinical relevance.

PATIENTS AND METHODS

Histopathologic glossary. *Fully benign lymphoid infiltrate.* Fully benign lymphoid or lymphoplasmacellular infiltrates are present within the stroma, with or without an associated lymphoid follicular structure, without Ig light chain restriction in B cells, and without any displays of aggressive behavior (e.g., lymphoepithelial aggressiveness in mucosa-associated lymphoid tissue [MALT] sites). With reference to the gastric microenvironment, we used the more specific terms, grade 1 or grade 2 gastric lymphoid infiltrates, as described by Wotherspoon et al (18).

Lymphoproliferative lesion. Heavy lymphoid infiltration is evident in lymphoproliferative lesions. Areas of Ig light chain restriction in B cells may or may not be present, and there is the usual presence of centrocyte-like (CCL) cells. A more or less pronounced lymphoepithelial aggressiveness is present in MALT sites, without definite evidence of B cell malignancy. With reference to the parotid, gastric, and nodal microenvironments, we used the more specific terms, myoepithelial sialadenitis (MESA) (3,4,16,17), grade 3 or grade 4 gastric lymphoid infiltrates (18), and atypical lymphoproliferative disorder (13,14), respectively. Patients defined as having an SS-associated lymphoproliferative disorder had to show pathologic evidence of a lymphoproliferative lesion.

Lymphoma. Diagnosis of a lymphoma requires unequivocal evidence of a frank B cell lymphoma according to currently accepted criteria (15–17). With particular reference to primary salivary gland lymphoma, which usually arises in the background of MESA, the diffuse or multifocal effacement and destruction of much of the salivary gland parenchyma by a polymorphic lymphoid infiltrate, mainly consisting of a population of CCL cells, should be observed. The characteristic neoplastic cell is a small-to-medium–sized lymphocyte, termed CCL, with irregular nuclear contours; it resembles a small cleaved follicular center cell, but with a moderate amount of pale-staining cytoplasm. The morphology of this CCL cell is somewhat variable. Monotypic plasma cells are also present. The CCL cells surround prominent follicles, which are a constant feature of the lesion, and invade the glandular epithelium. The epithelial involvement may be so extensive that, often, the epithelium is difficult to recognize without specific immunostaining techniques.

Patients with lymphoproliferative disorder. Six consecutive patients attending the Rheumatic Disease Unit of the University of Udine were studied. These 6 patients had definite primary SS (19) as well as a diagnosis of SS-related lymphoproliferative disorder (Table 1). All these patients had histopathologic evidence of lymphoproliferative lesions, but not of a frank lymphoma associated with SS. They all presented with persistent parotid swelling, i.e., duration of at least 2 months, with no phases of spontaneous regression. In our experience, this is the subgroup of SS patients in which the issue of putative progression toward B cell malignancy arises more frequently (6).

All the patients reported having asthenia and arthralgia. Weight loss (>3 kg in the previous 3 months) was recorded for 4 patients (patients 1, 3, 4, and 6). All had extraglandular features (Table 1) and detectable serum antinuclear antibodies (by indirect immunofluorescence on HEp-2 cells). Five were anti–SS-A positive and 3 were both anti–SS-A and anti–SS-B positive (by enzyme-linked immunosorbent assay). No patient showed a serum M component on electrophoresis and immunofixation.

All the patients underwent incisional parotid biopsy (20), preceded by salivary gland echography (21). Half of the biopsy samples were snap-frozen in liquid nitrogen and stored at −80°C; the other half were fixed in Bouin or formalin and embedded in paraffin. Parotid lymphoproliferative lesions, i.e., an indication of MESA (3,4,16,17), were observed in all tissue samples by conventional hematoxylin and eosin staining, but no sample showed pathologic features of frank primary salivary gland lymphoma. Further histologic analysis and immunohistologic characterization of cryopreserved and paraffin-embedded tissue sections included the study of B cell (anti-CD20), T cell (anti-CD3, anti-CD4, and anti-CD8), plasma cell

Table 1. Clinical, histopathologic, and molecular data on patients with Sjögren's syndrome and an associated lymphoproliferative disorder*

Patient/ age/sex	Systemic features	Tissue biopsy, date	Pathologic diagnosis	Detection of B cell clonality		
				κ/λ phenotype	Southern blot	Radioactive PCR
1/67/F	AN, AR, CR, N	R-parotid, Sept '94	MESA	−	+	+ (1)†
		Lymph node (R-inguinal), Aug '94	Atypical lymphoproliferation	+κ	+	+ (1)†
		Stomach, Sept '94	Antrum: WIG grade 3, HP+	−	ND	−
			Fundus: WIG grade 5, HP+	+κ	ND	+ (1)†
		Lymph node (R-inguinal), Mar '95	Atypical lymphoproliferation	+κ	+	+ (1)†
		Stomach, Mar '95	Antrum: WIG grade 1, HP−	−	ND	−
			Fundus: WIG grade 1, HP−	−	ND	+ (1)†
		Stomach, Jul '96	Antrum, corpus, and fundus: NHL, diffuse large B cell, HP−	+κ	ND	+ (1)†
2/46/F	AR	R-parotid, Jul '93	MESA	−	+	+ (2)†
		Stomach, Jul '93	Antrum: WIG grade 1, HP−	−	ND	−
		R-parotid, May '94	MESA	−	+	+ (2)†
3/41/F	AN, AR, ILD, IN	L-parotid, Jul '93	MESA	−	−	−
		Stomach, Jul '93	Antrum: WIG grade 3, HP−	−	ND	+ (5)
		Kidney, Jul '93	IN, mesangial GN	−	ND	−
		L-parotid, Feb '94	MESA	−	−	+ (4)
		Stomach, Jan '94	Antrum: WIG grade 3, HP−	−	ND	−
		Stomach, Jul '94	Antrum: WIG grade 3, HP−	−	ND	+ (1)
4/57/F	AR, CV, CR, ILD, N, P	L-parotid, Apr '93	MESA	−	+	+ (3)
		Stomach, Apr '93	Antrum: WIG grade 1, HP+	−	ND	+ (1)
		Stomach, Feb '94	Antrum: WIG grade 3, HP+	−	ND	+ (1)
5/43/F	AR, ILD	L-parotid, Jul '93	MESA	−	−	+ (2)†
		Stomach, Jul '93	Antrum: WIG grade 1, HP+	−	ND	−
		L-parotid, Jan '95	MESA	+κ	+	+ (2)†
		L-parotid, Jul '96	MESA	ND	ND	+ (2)†
6/56/F	AR, CV, IC	L-parotid, Jan '95	MESA	−	−	+ (6)
		Stomach, Jan '95	Antrum: WIG grade 1, HP−	−	ND	+ (4)

* PCR = polymerase chain reaction; AN = anemia; AR = arthritis/arthralgia; CR = cryoglobulinemia; N = peripheral neuropathy; MESA = myoepithelial sialadenitis; WIG = grading of gastric lymphoid infiltration according to Wotherspoon et al (see ref. 18); HP+ or HP− = positivity or negativity for *Helicobacter pylori* infection in the gastric mucosa; ND = not determined; NHL = non-Hodgkin's lymphoma; ILD = interstitial lung disease; IN = interstitial nephritis; GN = glomerulonephritis; CV = cutaneous vasculitis; P = purpura; IC = interstitial cystitis.
† These dominant bands appeared to be of the same molecular weight, and thus were further analyzed by PCR–single-strand conformation polymorphism and DNA sequencing.

(anti-CD38), natural killer cell (anti-CD57), granulocyte (anti-CD15, or endogenous peroxidase), and histiocyte/macrophage-associated (anti-CD68) antigens, as well as the restriction of surface κ/λ Ig light chains (22).

Synchronous biopsy specimens from other tissues were obtained from all patients because of concomitant clinical manifestations (i.e., gastric discomfort in all patients, active nephritis in 1 patient [patient 3], and lymphadenopathy in 1 patient [patient 1]). Biopsies of metachronous tissue from the same organ (parotid, stomach, or lymph node) were performed in some patients because of previous histopathologic lesions of major severity, and/or because of persistent or recurrent disease manifestations such as parotid swelling, gastric discomfort, or lymphadenopathy (parotid biopsy was repeated in 3 patients, lymph node biopsy in 1 patient, and gastric biopsy in 3 patients) (Table 1). *Helicobacter pylori* (HP) infection in the gastric mucosa was assessed by modified Giemsa staining and rapid urease test (CLO test; Delta West, Bentley, Australia).

At baseline in the present study, all 6 patients were untreated. The following treatments were started after the biopsies of synchronous tissue were performed, and were continued until the biopsies of metachronous tissue were performed. As part of an ongoing trial, interferon-α (IFNα), 3×10^6 international units, was administered 3 times per week to patients 1, 2, 4, and 5 (23). Corticosteroids, in 2 consecutive 6-month cycles were administered to patient 3 due to renal involvement (24). The corticosteroid regimen was as follows: 6-methylprednisolone, 250 mg/day for 3 days and 125 mg/day for another 3 days, then 25 mg/day of prednisone, with a tapering of 5 mg every month until the minimum dose of 5 mg/day was reached (first cycle, months 1–6; second cycle, months 7–12). Two patients with gastric infection by HP (patients 1 and 4) were treated with 1 course of amoxicillin, 500 mg 4 times per day, plus omeprazole, 20 mg 2 times per day, for 14 days, then with omeprazole 20 mg/day for 30 additional days. All the patients were then strictly followed up for the appearance of lymphoma.

Patients with fully benign lymphoid infiltrates. Fully benign lymphoid infiltrates in tissue obtained from 11 other patients were also investigated by molecular analyses. These 11 patients (mean ± SD age 60.8 ± 9.3 years) had a definite diagnosis of primary SS (19), had no serum M component, had no associated lymphoproliferative disorder, and were all untreated at study entry. The tissue lesions were from a site where B cell NHL may develop, i.e., the gastric mucosa. Gastric lesions were graded according to the system of Wotherspoon et al (18). The lymphoid infiltration, although well represented, was neither diffuse nor aggressive toward the glandular or foveolar epithelium (18). Samples with poor lymphoid infiltrates (grade 0) (18) were excluded, since it has been hypothesized that false-positive molecular results may occur in such cases (25).

Molecular studies. DNA was recovered from fresh-frozen samples by standard procedures (26). For synchronous and metachronous biopsy specimens from the same patient, extractions were performed at different times to avoid the risk of cross-contamination. These samples were representative of the whole tissue lesion, as shown by comparison with the results of hematoxylin and eosin staining of paraffin-embedded and fresh-frozen sections. For each patient, an average of 3 gastric biopsy samples (usually from the antral mucosa) were subjected to DNA extraction, whereas an average of 7 biopsy specimens from the same gastric region were devoted to histopathologic studies. Much higher amounts of DNA were obtained from the parotid and lymph node biopsy samples, whereas lower amounts (~10–50 μg) were obtained from the gastric and kidney biopsy samples.

Southern blot analysis. Southern blot was performed, as previously described (26), on tissue from which rather high amounts of DNA could be obtained, i.e., the parotid MESA samples from all patients and the lymph node biopsy samples from patient 1. DNA from most gastric biopsy samples, as well as from the kidney biopsy sample of patient 5, was not analyzed by Southern blot, since the amount of DNA was hardly sufficient for evaluation and no additional DNA would have been available for molecular studies. Southern blot could be performed on 5 of the 11 fully benign gastric lesions in tissue from SS patients with no suspected lymphoproliferation. These analyses were limited to 2 restriction enzymes, *Hind* III and *Eco* RI.

Polymerase chain reaction (PCR). All the tissues were analyzed by PCR for B cell clonal expansion, using 2 different seminested protocols of amplification (6). In the first protocol, the upstream primer was complementary to the third framework V region (FR3) of the IgH gene, whereas in the second protocol, the upstream primer was complementary to the second framework V region (FR2). In both protocols, the downstream primer was the same and was directed to an outer conserved region of the IgH J region in the first round of amplification, and to an inner conserved sequence of the same J region in the second round (6). Each sample was tested in triplicate, and any positive or negative result was confirmed by at least 2 separate PCR experiments. The sensitivity of this V-D-J–PCR approach was superior to that of Southern blot under standard conditions (0.5–1% versus 5%) (6,26).

Nonradioactive PCR. First, a "cold" nonradioactive PCR was performed (6). At the end of the second-round amplification, 20 μl of reaction mixtures was analyzed by electrophoresis in 5% (FR3 protocol) or 3% (FR2 protocol) agarose gel in Tris-borate-EDTA buffer, stained with ethidium bromide, and optically evaluated by ultraviolet transillumination. Clonal expansion was indicated by 1 or more dominant bands within the predicted size range, and a fully polyclonal pattern was evident by a smear with no specific dominant bands.

Positive and negative controls were included. The sensitivity of the technique was checked by amplification of serial dilutions of DNA from clonal B cells admixed with DNA from polyclonal B cells (the limit of detection of a discrete band was 0.5–1%, with a slightly higher sensitivity for the FR3 protocol) (6).

Radioactive PCR. Another approach was represented by radioactive PCR in the second round of amplification, in both the FR2 and FR3 protocols. Radioactive PCR was performed using a labeled nucleotide, i.e., α^{33}P-labeled dATP (Amersham, Bucks, UK). The radioactive PCR products were then subjected to a long-run (20–40-cm) electrophoresis on a nondenaturing 8% (FR3 protocol) or 6% (FR2 protocol) polyacrylamide gel. A ^{33}P-labeled primer, followed by electrophoresis of radioactive PCR products on acrylamide-urea denaturing gel, was also used in selected cases (data not shown) (27). The conditions of amplification were the same as those previously reported for the second-round amplification of "cold" nonradioactive PCR (6). Dried gels were then autoradiographed using a beta-max film (Amersham). Exposure was optimized to obtain a sensitivity similar to that of nonradioactive PCR analyzed on agarose gel.

PCR–single-strand conformation polymorphism (SSCP) and DNA sequence analysis to assess clonal identity. When the radioactive PCR products from synchronous or metachronous biopsy specimens appeared to be roughly of the same molecular weight (and, then, possibly related to the same B cell clone), the subsequent steps taken included SSCP analysis of radioactive V-D-J–PCR products, and DNA sequencing, as previously described (28,29).

In PCR-SSCP evaluations, the position at which the strands are immobilized in the gel is a function of the conformation of the strand, which, in turn, is determined by its nucleotide sequence. The technique is sensitive enough to detect a single base change in a fragment of several hundred bases, and has therefore been proposed to detect point mutations before sequencing (28,29). In contrast, identical SSCP patterns in different PCR products imply an identical nucleotide sequence. B cell clonal identity may then be detected.

For DNA sequencing, nonradioactive PCR products from synchronous or metachronous biopsy specimens were size fractioned by gel electrophoresis on agarose or acrylamide gel. The dominant bands were cut from the gel. In 1 case DNA was recovered and reamplified by a second round of seminested nonradioactive V-D-J–PCR, to enhance for the sequence. PCR products (single bands) were then purified from agarose gel and subjected to direct DNA sequencing using sense and antisense primers (28,29). A negative control (fully polyclonal) V-D-J–PCR product from an SS-unrelated reactive lymphadenopathy was included in the procedure to exclude possible false-positive results due to DNA contamination.

Use of clonospecific probes to assess clonal persistence or dissemination. To increase the sensitivity of assessing the ongoing expansion or dissemination and local expansion of the

same B cell clone in synchronous and metachronous tissue lesions, the highly sensitive molecular approach that requires the generation of clonospecific oligoprobes was used (30). This approach is currently used to sensitively monitor residual disease in patients with leukemia or lymphoma, detecting 1 malignant cell in 10^4–10^6 normal cells (30). Only those SS samples for which V-D-J–DNA sequence analysis had been performed, and that lacked evidence of either clonal persistence or dissemination by the previous molecular approaches, were studied. The highly sensitive clonospecific probe approach should detect a very low number of clonally expanded cells, below the limit of detection by the previous approaches.

Clonospecific oligoprobes were synthesized based on the V-D-J–DNA sequence of the dominant B cell clone, expanded in 1 single lymphoproliferative lesion (parotid lesion). For any single V-D-J–DNA sequence, 2 different clonospecific oligoprobes were designed, on either the V-N-D or the D-N-J junctional sequence. The clonospecific oligoprobe was labeled at the 5′ end by T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and γ^{32}P-labeled dATP (Amersham). In each experiment, nonradioactive V-D-J–PCR products in the different synchronous and metachronous biopsy tissues from the same patient were blotted on a nylon membrane (Biodine-B; Pall, Portsmouth, UK), hybridized for 12 hours with 1 single radiolabeled clonospecific oligoprobe, and washed with 6× saline–sodium citrate, 1% sodium pyrophosphate. Hybridization and washing temperatures were optimized for any single oligoprobe under stringent conditions. Sensitivity was tested in serial dilution experiments, and specificity was assessed by including proper clonal or polyclonal V-D-J–PCR products from control patients.

RESULTS

Clinical followup of patients with lymphoproliferative disorder. At present, 5 of the 6 study patients with lymphoproliferative disorder are considered to be well. Parotid swelling has completely regressed in 4 of these patients (patients 2, 3, 4, and 6). In December 1995, treatment with IFNα was suspended in patient 2, while patients 4 and 5 are still under treatment. Renal function has normalized in patient 3, who has been taking 5 mg/day of prednisone since July 1994. In July 1996, patient 5 experienced a new episode of bilateral parotid swelling. The parotid biopsy was then repeated, revealing no evidence of lymphoma progression.

In contrast, a high-grade malignant lymphoma has very recently complicated the disease course of patient 1. From March 1995 to June 1996, patient 1 was observed to be well (IFNα therapy was suspended in February 1995 after the development of nephritis and serositis, which were then treated with corticosteroids). In October 1995, computed tomography scanning showed a definite decrease in lymphadenopathy. In July 1996, parotid swelling had completely regressed, but the patient reported having dyspepsia and vomiting. A second endoscopic examination and gastric biopsy documented a high-grade gastric B cell lymphoma. Computed tomography scanning showed a recurrence of lymph node enlargement.

Histopathologic analysis. Among the parotid biopsy samples containing MESA lesions, 1 (from patient 6) showed heavy, but focal, lymphoid infiltration. The overall structure of the salivary gland was intact, but lymphoid nodules surrounding dilated ducts were present. These nodules consisted of a population of small cells exhibiting a B cell phenotype with no Ig light chain restriction. T cells were numerous and a polytypic population of plasma cells was also present. Lymphoepithelial aggressiveness by CCL cells was less pronounced than in the other MESA samples, without any glandular effacement. Another parotid biopsy sample (from patient 3) showed focal lymphoid infiltration, but also showed areas of extensive lymphoid infiltration. Similar features were recognizable on a second parotid biopsy of tissue from the same patient (Table 1).

The other 4 samples (from patients 1, 2, 4, and 5) showed MESA with dense and diffuse lymphoid infiltration (Table 1). In tissue from patients 1, 4, and 5, the architecture of the salivary gland was, in part, effaced by a lymphoid population containing epimyoepithelial islands infiltrated by lymphocytes. Immunohistochemical studies identified the overwhelming B cell nature of the infiltrate, but T cells were also abundant. Staining for Ig light chains revealed the polytypic nature of B cells, and highlighted a polytypic plasma cell population. In the fourth sample (from patient 2), an expanded population of CCL cells formed a "halo" around the epimyoepithelial islands, sparing the reactive follicles. The architecture of the salivary gland was substantially preserved, and no Ig light chain restriction was observed.

In 1 of these 4 samples (from patient 5), the second parotid biopsy identified histologic features that were essentially superimposable with those identified on the first biopsy, except for the CCL cell component. These cells closely associated with epimyoepithelial islands, a number of which (approximately one-third) were partially disrupted. An increased κ:λ ratio (5:1) could be demonstrated in the CCL cells. However, the whole picture was inconsistent with a frank B cell lymphoma (16,17). In a recent parotid biopsy, performed on patient 5 after 13 months of additional followup, the histopathologic picture was markedly improved, showing a MESA with mild and occasional lymphoepithelial aggressiveness (the κ:λ ratio could not be evaluated due to the scarcity of biopsy tissue). Patient 3 showed a concomitant renal lymphoproliferative le-

sion, i.e., heavy interstitial lymphoid infiltration, lymphoepithelial aggressiveness, and tubular damage, plus mild mesangial glomerulopathy with IgM and C3 deposits.

In all 6 patients, the biopsy samples showed well-represented MALT in the gastric mucosa. Concomitant infection by HP was detected in tissue from 3 patients (Table 1). The gastric lesions were classified, by histopathologic and immunohistochemical analyses, according to the grading system of Wotherspoon et al (18). These lesions ranged from fully benign infiltrates (grades 1 or 2 in tissue from patients 2, 5, and 6) to florid infiltration with lymphoepithelial lesion (grade 3 in tissue from patients 3 and 4). An extensive gastric infiltrate compatible with a low-grade lymphoma (grade 5) (18) was observed in the gastric fundus tissue of patient 1. The lymphoid infiltrate regressed to grade 1 chronic gastritis after eradication of HP infection (31).

Nodal atypical lymphoproliferation with diffuse pseudoobliterative pattern (13,14), but not a lymphoma, was concomitantly observed in the sample from patient 1 (31). Pseudoobliteration of the lymph node architecture was present, with partially discernible compartments showing some preserved germinal centers and diffuse lymphoplasmacytoid and immunoblastic infiltration, associated with clusters of epithelioid cells and occasional CCL cells. Immunoblasts showed only occasional nuclear atypia, and consisted of B cells (CD20-reactive) and T cells (CD3-reactive) in variable proportions. The pathologic diagnosis was unchanged following a biopsy of metachronous lymph node tissue (31). In July 1996, a malignant B cell lymphoma was again documented in the stomach. However, it proved to be a diffuse large cell lymphoma, negative for HP, that involved the gastric fundus, corpus, and antrum, and caused gastric stenosis.

Molecular analyses. Four of 6 parotid biopsy samples with MESA (from patients 1, 2, 4, and 5) showed evidence of B cell clonal expansion by Southern blot (Table 1). In patient 1, both the parotid and the 2 lymph node biopsy specimens were positive by Southern blot, and rearranged bands appeared to be of the same molecular weight. In patient 2, both the first and the second parotid biopsy specimens were positive by Southern blot, but different rearranged bands were noted in the 2 samples. In patient 5, the first parotid biopsy yielded negative results by Southern blot, whereas positivity was noted on the parotid biopsy of metachronous tissue, concomitant with evidence of κ light chain restriction (no light chain restriction was evident on the first parotid biopsy). Southern blot was more sensitive than κ/λ immunophenotyping in the detection of B cell clonality, since the tissue samples showing a light chain restriction by immunophenotyping were also positive by Southern blot, while the converse was not observed.

Evaluation by V-D-J–PCR proved to be even more sensitive than Southern blot in the detection of B cell clonal expansion, since B cell clonal expansion was demonstrated in the parotid MESA samples from all 6 SS patients (only the first parotid biopsy sample from patient 3 showed no clonal expansion by either Southern blot or V-D-J–PCR) (Table 1). B cell clonality was demonstrated by V-D-J–PCR in the gastric biopsy samples from 4 of these patients, as well as in both lymph node biopsy specimens from patient 1, but not in the kidney biopsy sample from patient 3 (Table 1). Possible false positivities were definitely excluded by the concomitant negative results achieved in negative controls (i.e., PCR reactions without DNA or with DNA from fully B cell polyclonal lesions that were SS-unrelated).

Overall, the detection of B cell clonality by either nonradioactive or radioactive V-D-J–PCR (Figure 1) provided concordant positive or negative results. In tissue from 3 patients (gastric samples from patients 4 and 5 and the first parotid biopsy sample from patient 3), uncertain positivity by the nonradioactive approach could be defined as either positivity or negativity by the radioactive approach (Table 1). In some instances, the radioactive PCR approach demonstrated that B cell expansions were oligoclonal (more than 2 dominant bands) rather than monoclonal (Table 1) (27). Such an oligoclonal pattern was established in the parotid biopsy samples from patients 3, 4, and 6, as well as in the gastric samples from patients 3 and 6.

Biopsies of synchronous tissue. Dominant bands apparently of the same molecular weight were visualized by both nonradioactive and radioactive V-D-J–PCR in synchronous tissue (lymph node, parotid, and gastric fundus biopsy specimens) from only 1 patient (patient 1) (Table 1), thus suggesting the possible dissemination of the same B cell clone (31).

Among the remaining samples, dominant bands of different molecular weight were visualized by radioactive PCR in parotid and gastric biopsies of synchronous tissue from patients 3, 4, and 6, whereas in tissue from patients 2, 3, and 5, parotid positivity versus gastric negativity, or the reverse, was observed (Table 1). This finding is consistent with the expansion of different dominant B cell clones in different tissues from the same patient.

Biopsies of metachronous tissue. Bands apparently of the same molecular weight were observed in metachronous biopsy specimens from the same affected

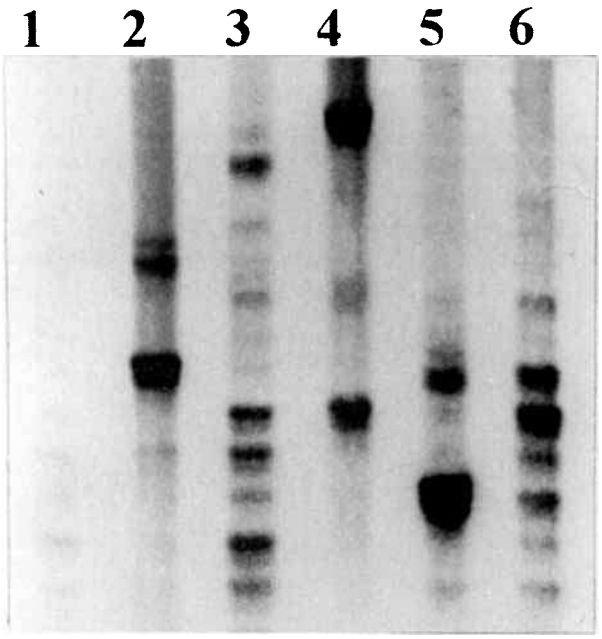


Figure 1. Assessment of B cell clonality in patients with Sjögren's syndrome (SS), by radioactive polymerase chain reaction (FR3 protocol). Lane 1, A polyclonal pattern with no dominant band (gastric biopsy tissue from SS patient 2). Lanes 2, 4, and 5, A monoclonal pattern (first parotid biopsy samples from SS patients 2 and 5 and an SS-unrelated malignant B cell lymphoma specimen, respectively). Lanes 3 and 6, An oligoclonal pattern with more than 2 dominant bands (chronic gastritis tissue from an SS patient and reactive lymphadenopathy tissue from a patient without SS, respectively).

tissue in patient 1 (in the right inguinal lymph nodes and gastric fundus, and, recently, in the gastric fundus, corpus, and antrum, which were involved by high-grade lymphoma) (31). Bands of the same molecular weight were also observed in tissue from 2 of the 3 patients who received second parotid biopsies, i.e., from patient 2 (limited to PCR products amplified by the FR3 protocol) and from patient 5 (Figure 2) (data not shown with regard to a third parotid biopsy sample from patient 5).

In patient 2, different B cell clones were expanded in the metachronous tissue from the right parotid biopsy, and no B cell clonality was detected in the gastric mucosa. A dominant band (monoclonal pattern), apparently of the same molecular weight, was amplified in both the first and the second parotid biopsy samples, whereas no dominant band (polyclonal pattern) was evident in the gastric biopsy tissue (patient 2, Figures 2A and B).

In patient 5, the same B cell clone was expanded in the metachronous tissue from the left parotid biopsy, whereas no B cell clonality was detected in the gastric mucosa. Two dominant bands (monoclonal pattern), apparently of the same molecular weight, were amplified in both the first and the second parotid biopsy samples, whereas no dominant band (polyclonal pattern) was shown in the gastric biopsy tissue (patient 5, Figures 2A and B).

Discordant results (positivity versus negativity or dominant bands of different molecular weight) were established by radioactive PCR in parotid biopsies of metachronous tissue from patient 2 (limited to PCR products amplified by the FR2 protocol) and from patient 3, as well as in gastric biopsies of metachronous tissue from patients 3 and 4.

Assessment of B cell clonal identity by PCR-SSCP and DNA sequence analyses. PCR-SSCP analysis and DNA sequencing of the V-D-J–amplified products were performed; these analyses were limited to tissue specimens from patients 1, 2, and 5. In fact, only in these patients did the radioactive V-D-J–PCR products from synchronous or metachronous tissue appear to be roughly of the same molecular weight, and then, possibly, were related to the same B cell clone. Thus, it was necessary to definitely assess clonal identity.

Analysis by SSCP indicated clonal identity (identical banding patterns, i.e., identical nucleotide sequences) in synchronous and metachronous parotid, lymph node, and gastric (fundus) biopsy specimens from patient 1 (31), including the recently developed gastric high-grade lymphoma (in the fundus, corpus, and antrum) (data not shown). SSCP analysis also identified the same B cell clone in both the first and the second metachronous parotid biopsy specimens from patient 5 (Figure 2C) (data not shown with regard to a third parotid biopsy sample).

In contrast, SSCP analysis of radioactive V-D-J–PCR products, amplified by the FR3 protocol, revealed a different banding pattern in the metachronous parotid biopsy specimen from patient 2 (Figure 2C). This might indicate either the expansion of 2 completely different dominant clones in the 2 metachronous parotid biopsy samples (as also suggested by the results of the FR2 protocol), or the ongoing expansion of the same dominant clone with limited nucleotide sequence diversity (intraclonal diversity, antigen-driven somatic mutations). No band could be visualized by SSCP with regard to antral mucosa specimens from patients 1, 2, or 5.

Where identical SSCP banding patterns had been observed, i.e., in tissue from patients 1 and 5, identical nucleotide sequences (clonal identity) were subsequently confirmed by DNA sequencing (patient 5, Figure 2D) (data not shown with regard to patient 1 and the

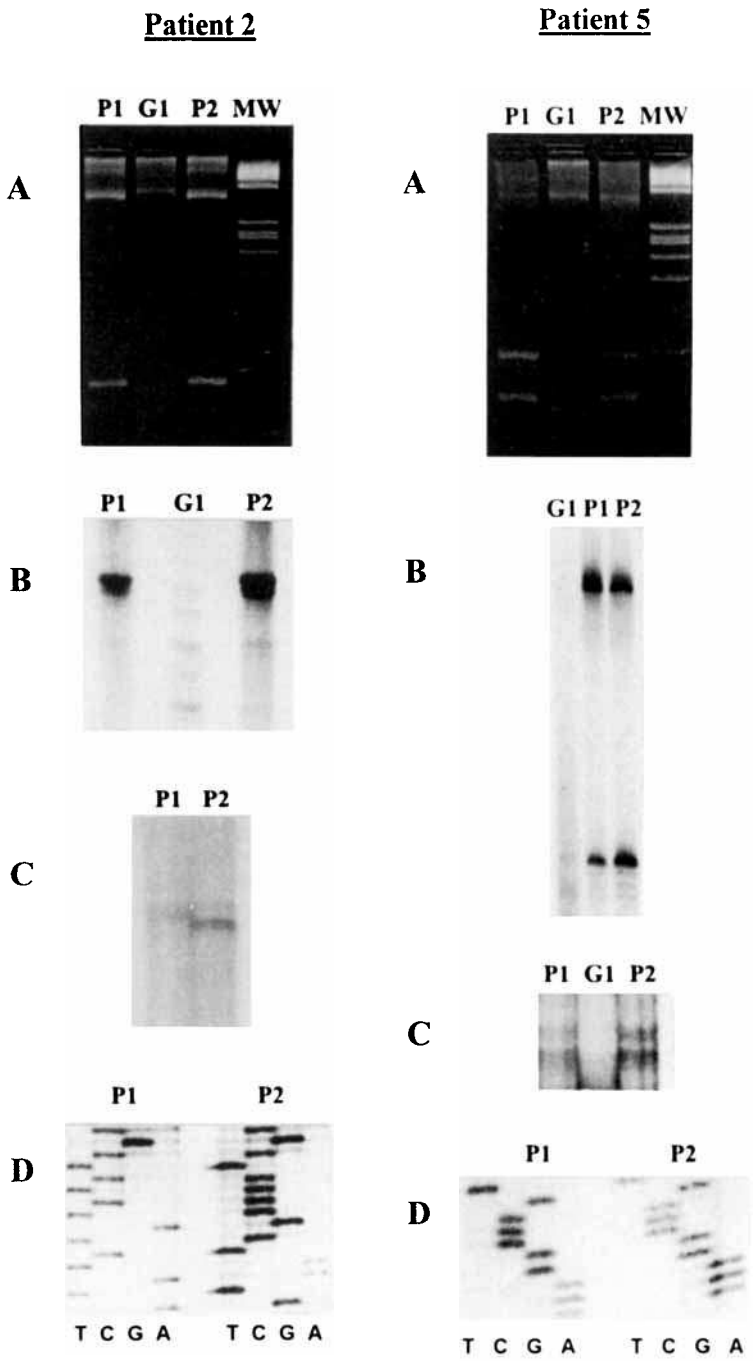


Figure 2. Assessment of B cell clonal expansion in tissue from Sjögren's syndrome patients with parotid lymphoproliferative lesion (patients 2 and 5), by multiple molecular analyses. Lane P1, Parotid biopsy sample at baseline. Lane G1, Gastric (chronic gastritis) biopsy sample at baseline. Lane P2, Metachronous parotid biopsy sample during followup (see also Table 1). **A,** Agarose gel stained with ethidium bromide, showing nonradioactive V-D-J–polymerase chain reaction (PCR) products (FR3 protocol) from synchronous and metachronous biopsy samples. **B,** Dried polyacrylamide gel, showing radioactive V-D-J–PCR products (FR3 protocol). **C,** Single-strand conformation polymorphism analysis of radioactive V-D-J–PCR products (FR3 protocol). **D,** DNA sequencing of the dominant band of V-D-J–PCR products (FR3 protocol).

third parotid biopsy sample from patient 5) (31). In patient 5, the complete V-D-J sequence from the FR3 protocol, excluding the primers used for sequencing, was 5′-GCAAGA(V)AGGGGAGGGGCCCCG(N)TTATGACTATGATAACAGTGGATATTAC(DXP4)GGGCCTT(N)TACTCCTGAGCTATGGACGT(J6)-3′. The identical sequences of the DXP4-N-J6 recombination site in both parotid biopsy samples are shown in Figure 2D (patient 5). Antisense sequences were related to the dominant band of higher molecular weight in both samples.

In contrast, entirely different V-D-J sequences (completely different dominant clones, using different V, D, and J genes) (32,33) were demonstrated by V-D-J–PCR in the dominant bands of the metachronous parotid biopsy tissue from patient 2, in which a different SSCP banding pattern had been noted (patient 2, Figure 2D). Both the FR2- and the FR3-amplified V-D-J–PCR products in the metachronous tissue from patient 2 were sequenced, and the same 2 different dominant clones were identified. While the number of basepairs differed in the dominant bands of the metachronous tissue amplified by the FR2 protocol (data not shown), the number of basepairs was identical in the dominant bands amplified by the FR3 protocol (patient 2, Figure 2D).

In the first parotid biopsy sample from patient 2, the expanded B cell clone had rearranged the DLR2 and J2 genes (the complete V-D-J sequence from the FR3 protocol, excluding the primers used for sequencing, was 5′-GCGAGA[V]GATA[N]GATATTGTAGTGGTGGTAGCTGCT[DLR2]TTG[N]ACTGGTACTTCGATCT [J2]-3′), whereas the clone that was overexpanded in the second parotid biopsy sample used the DXP4 and J3 genes (the complete V-D-J sequence from the FR3 protocol, excluding the primers used for sequencing, was 5′-GCGAG[V]GGGCG[N]ATTACTATGATAGTAGTGATT[DXP4]CTTTCCA[N]-TGATGTTTTTGATGT[J3]-3′). The sequences of the different V-N-DLR2 (first parotid biopsy sample) and V-N-DXP4 (second parotid biopsy sample) recombination sites are shown in Figure 2D (patient 2) (antisense sequences).

Analysis of clonal persistence or dissemination using clonospecific probes. In the samples from patients 2 and 5, clonospecific oligoprobes were designed on the V-D-J–DNA sequences of the dominant B cell clones that were expanded in the parotid lesions. In the first parotid biopsy sample from patient 2, 2 clonospecific oligoprobes were designed on either the V-N-D or the D-N-J junctional sequences of the expanded clone (probes P1.V-N-D and P1.D-N-J, respectively), and in the second parotid biopsy sample, 2 additional clonospecific probes were designed on either the V-N-D or the D-N-J junctional sequences of the different expanded B cell clones (probes P2.V-N-D and P2.D-N-J, respectively) (Figure 3). In patient 5, 2 clonospecific oligoprobes were designed on either the V-N-D or the D-N-J junctional sequences of the expanded clones, which were the same in both the first and the second parotid biopsy samples (probes P1/2.V-N-D and P1/2.D-N-J, respectively) (Figure 3).

In the samples from patient 2, the clonospecific probe approach proved to be highly sensitive when used to verify 1) whether the dominant clone that was overexpanded in the first parotid biopsy sample was still expanded in the second parotid biopsy sample (although reduced in size), and whether the dominant clone that was overexpanded in the second parotid biopsy sample was already expanded in the first parotid biopsy sample; and 2) whether either of the 2 parotid clones, or both, were also expanded in the gastric mucosa. In the samples from patient 5, the clonospecific probe approach also proved to be sensitive when used to verify whether the same B cell clone, which was expanded in both the first and the second parotid biopsy samples, was also expanded in the gastric mucosa. The previous results obtained by both radioactive and nonradioactive V-D-J–PCR had excluded these possibilities (Figure 1).

As expected (30), and as shown in Figure 3, the clonospecific oligoprobe approach allowed us to test these possibilities by increasing the sensitivity by 2 orders of magnitude in all the experiments (from 1% to 0.01%, i.e., B cell clonal expansion accounted for 0.01% of the whole cells in the tissue samples). The negative results of the previous molecular tests were confirmed in all the different experiments by using any single clonospecific probe.

Figure 3 shows the results using probe P1.V-N-D in the samples from patient 2, and probe P1/2.D-N-J in the samples from patient 5. In the tissue from patient 2, identical results were obtained by using the P1.D-N-J oligoprobe (5′-TAGCTGCTTTGACTGGTACT-3′) (data not shown). Thus, the B cell clone that was expanded in the first parotid biopsy tissue was neither disseminated and locally overexpanded in the gastric mucosa, nor was it detectable in the second parotid biopsy tissue. Similar results were also observed by the use of both clonospecific oligoprobes for the B cell clone that was expanded in the second parotid biopsy tissue (P2.V-N-D 5′-GCGAGGGGCGATTACTATGATA-3′; P2.D-N-J 5′-GTGATTCTTTCCATGATGT-3′), which was detected neither in the first parotid biopsy tissue nor in the gastric mucosa (data not shown).

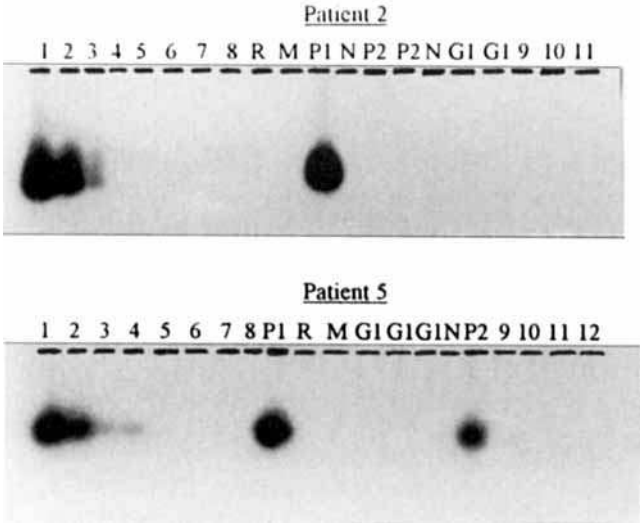


Figure 3. Assessment of clonal persistence and dissemination, by the highly sensitive clonospecific oligoprobe approach, in the lesions of synchronous and metachronous parotid and gastric tissues from Sjögren's syndrome [SS] patients 2 and 5. The following lanes are the same for both patients. Lane P1, Parotid biopsy sample at baseline. Lane P2, Metachronous parotid biopsy sample during followup (tested in duplicate for patient 2). Lane G1, Gastric (chronic gastritis) biopsy sample at baseline (tested in duplicate for patient 2 and in triplicate for patient 5) (see also Table 1). Lane 9, SS-unrelated reactive lymphadenopathy tissue (control). Lane 10, First lymph node biopsy tissue from patient 1 (control). Lane 11, SS-unrelated grade 1 chronic gastritis tissue (control). Lane N, Negative control without DNA. Lane M, Molecular weight markers. Patient 2: Clonospecific oligoprobe P1.V-N-D (5′-GCGAGAGATAGATATTGTA-3′) was used for hybridization. DNA from the P1 tissue was diluted with DNA from the P1 tissue of patient 3 (lane R), in the proportions 1:1 (lane 1), 1:10 (lane 2), 1:100 (lane 3), 1:250 (lane 4), 1:500 (lane 5), 1:1,000 (lane 6), 1:10,000 (lane 7), and 1:100,000 (lane 8). A positive signal was observed only in lanes 1–3 (100-fold increase in sensitivity) and in lane P1. Patient 5: Clonospecific oligoprobe P1/2.D-N-J (5′-ACGGGCCTTTACTCCTGAG-3′) was used for hybridization. DNA from the P1 tissue was diluted with DNA from the gastric biopsy tissue of a control patient (lane R), in the proportions 1:1 (lane 1), 1:10 (lane 2), 1:100 (lanes 3 and 4), 1:250 (lane 5), 1:500 (lane 6), 1:1,000 (lane 7), and 1:10,000 (lane 8). A positive signal was observed only in lanes 1–4 (100-fold increase in sensitivity) and in lanes P1 and P2.

In the tissue from patient 5, similar results were observed by the use of P1/2.V-N-D clonospecific oligoprobe (5′-AGGGGCCCCGTTATGA-3′) (data not shown), indicating that the B cell clone that was expanded in both the first and the second parotid biopsy tissues was not disseminated and locally overexpanded in the gastric mucosa. Thus, no gastric dissemination and local expansion of the parotid B cell clone(s) had occurred in either patient, and the 2 different parotid clones from patient 2 were never concomitantly detectable in the same parotid biopsy sample.

Patients with fully benign MALT lesions. Of the 11 samples containing fully benign gastric lymphoid infiltrates, obtained from patients with primary SS who had no associated lymphoproliferative disorder, 6 showed a grade 1 gastric involvement (chronic active gastritis), and 5 showed a grade 2 involvement (chronic active gastritis with florid lymphoid follicle formation), according to the grading system of Wotherspoon et al (18). In all samples, gastric MALT was well represented. Clusters of lymphocytes and/or lymphoid follicles were usually detected in the lamina propria, but lymphoepithelial aggressiveness was lacking and no κ/λ light chain restriction was noted by immunophenotyping (no evidence of gastric lymphoproliferation) (18). HP infection (previously untreated) was detected in 8 of the 11 samples.

Seven of the 11 specimens (63%) showed evidence of local B cell clonal expansion by radioactive V-D-J–PCR. More than 2 dominant bands (oligoclonal pattern) were noted in 6 of 7 samples. One sample showed a fully polyclonal pattern (smear with no dominant bands) by nonradioactive PCR run on agarose gel, whereas an oligoclonal pattern was shown by subsequent radioactive V-D-J–PCR. Uncertain positivity by nonradioactive PCR in 1 sample was later identified as B cell oligoclonal expansion. Finally, an oligoclonal pattern was also disclosed by radioactive PCR in 4 of 5 samples that showed only 1 or 2 dominant bands on agarose gel. No patient has developed a malignant lymphoma after a mean followup period of 3 years.

DISCUSSION

B cell lymphoproliferation is a key feature of SS, and lesions may range from fully benign to fully malignant (1–4). A better characterization of such evolution has important clinical implications. The evaluation of clonal B cell expansion is an important preliminary step to better investigate the pathobiologic events implicated in the different stages of progression and in different microenvironments. The comprehensive study of synchronous and metachronous biopsy samples from selected SS patients, by means of extensive and sensitive molecular analyses of B cell clonality, proved useful to better address this issue and to identify putative, more advanced stages of disease progression toward B cell malignancy.

Strict pathologic criteria remain, at present, the "conditio sine qua non" for the diagnosis of a frank B cell NHL in SS. The present clinical situation is patently unsatisfactory, since some borderline cases can be rather

difficult to define as either benign or low-grade malignant by histopathologic evaluation, and since the clinical behavior does not always correlate with the histopathologic findings. Disappointingly, definite oncogenic markers of malignant transformation have not been identified in SS-related lymphoproliferation. Furthermore, the simple detection of B cell clonality cannot be used as a criterion for the diagnosis of B cell malignancy, when a definite diagnosis by histopathologic evaluation alone is problematic. In fact, B cell clonal expansion is a common event in SS (whereas overt lymphoma develops in a minority of patients) (3,4). Thus, when also considering the currently accepted models of multistep B cell lymphomagenesis (34,35), the key question should not be whether B cell clonality is present in SS-related tissue lesions, but rather, whether the biology of the nonmalignant (i.e., lacking pathologic criteria for malignancy) B cell lymphoproliferative disorder more or less resembles that of a low-grade lymphoma (and then, whether the patient has a lymphoproliferative disorder that may be more or less advanced in progression toward B cell malignancy).

The evaluation of the persistence and dissemination of the same B cell clone, as well as the estimate of the "size" of the expanded B cell clone(s) during the disease, represented our initial approach to this issue. Multiple tissue lesions (synchronous from different tissues and metachronous from the same tissue) that lacked evidence of B cell malignancy by strict pathologic criteria were investigated. Only multiple and accurate analyses of B cell clonality (by Southern blot, V-D-J–PCR, SSCP, DNA sequencing, and hybridization with clonospecific oligoprobes) allowed us to reach definite conclusions.

It is the accurate clinical evaluation of the SS patient which, in our experience, indicates the tissues that might be involved in the lymphoproliferative disorder. Tissue biopsy should be primarily targeted to these sites. Heavy lymphoproliferation (when not a true lymphoma) is often observed by histopathologic evaluation (1–3,6,20).

By molecular analyses of synchronous biopsy specimens, the local overexpansion of the same B cell clone may be detected in multiple sites. This may not be a frequent event in SS-related lymphoproliferation, however, since it could be shown only for patient 1 in this study. As reported in detail and discussed more extensively elsewhere (31), HP infection played a role in the local boosting of B cell lymphoproliferation in the gastric mucosa in this patient. However, an underlying SS-associated B cell disorder was present, without any definite histopathologic evidence of malignancy, despite clonal persistence and dissemination (31). The possibility of a disseminated primary gastric low-grade lymphoma, SS-unrelated, was excluded by extensive clinical, histopathologic, and molecular evidence (31). Strikingly, after additional followup, this patient developed a high-grade gastric lymphoma, negative for HP, with diffuse large B cells.

In contrast, in the majority of SS patients with lymphoproliferative disorder, dominant bands of different molecular weight were observed in synchronous biopsy samples from different tissues, indicating different dominant B cell clones in different microenvironments. This supports the argument against an advanced, widespread clonal B cell lymphoproliferative disorder. Furthermore, the dissemination and local overexpansion of a single B cell clone in different MALT sites was definitely excluded in patients 2 and 5 by the highly sensitive clonospecific probe approach.

Multiple molecular analyses in metachronous tissue have shown that B cell clonal overexpansion is frequently multifocal and fluctuating in SS, since different clones predominated not only in different tissues (synchronous biopsy tissue), but also in the same affected tissue at different times (metachronous parotid biopsy tissue from patient 2, confirmed by the use of clonospecific probes, and from patient 3, and metachronous gastric tissue from patients 3 and 4). However, in tissue from 2 patients (patients 1 and 5), the overexpansion of the same B cell clone was definitely demonstrated in metachronous biopsy specimens from the same involved tissue. Positivity by Southern blot was also observed, indicating that the expanded B cell clone was well represented.

The possible persistence of the same B cell clone in metachronous MALT lesions has been suggested by recent studies and has been related to some pathologic features of low-grade malignancy of MALT (10,11). Furthermore, from a pathobiologic perspective, the ongoing expansion of a single dominant clone, as well as the clonal dissemination, is a feature of lymphoma. Two independent pathologists, after carefully reviewing the tissue lesions, ruled out the diagnosis of lymphoma in patients 1 (31) and 5. The risk of lymphoma progression was seriously considered, however, based on the molecular results. Importantly, a malignant lymphoma developed in 1 of the 2 patients (patient 1) after 16 months of followup. Thus, molecular criteria might be extremely useful in predicting lymphoma progression in cases where the sole histopathologic findings do not allow definite conclusions.

Due to the relevant clinical implications, this

issue should be accurately addressed by larger, integrated clinical, histopathologic, and molecular followup studies. High-risk situations should be unequivocally defined to avoid unnecessary chemo- or radiotherapeutic approaches and related complications. Definition of the oncogenic events involved in lymphoma progression (36–38) will also be crucial in the future, and definite markers of malignancy might be identified. Yet, only the concomitant definition of the role of local biologic networks (4,22) and (auto)antigenic triggering (4,31) would allow a fuller comprehension of B cell lymphomagenesis in SS, and would consistently improve the diagnostic and therapeutic approaches to this disease.

The results and implications of molecular analyses of B cell clonality should be clearly differentiated in the subset of fully benign lesions from SS patients without an associated lymphoproliferative disorder. Small B cell clonal expansions (e.g., negative by Southern blot/positive by PCR, usually oligoclonal by the radioactive V-D-J–PCR approach) occur quite frequently in fully benign lymphoid infiltrates involving tissues in which B cell NHL may develop, i.e., the gastric mucosa in this study. Our results regarding fully benign gastric lesions in patients with no associated lymphoproliferative disorder are consistent with the frequent detection of B cell clonality, by V-D-J–PCR, in the minor labial salivary glands of another series of SS patients with no associated lymphoproliferative disorder (39). Furthermore, clonal expansions are often observed in HP-associated chronic gastritis in nonautoimmune patients (40), but gastric lymphoma is not so frequent (41).

While these observations are important when investigating very early events in B cell lymphomagenesis in SS, on the whole it appears that small B cell clonalities, detected by highly sensitive molecular approaches in lesions with fully benign lymphoid infiltrates, may be of little clinical relevance. Therefore, molecular analyses should not be primarily targeted to such lesions in SS. Other investigators, however, have suggested that the dissemination of an overt malignant lymphoma could be highlighted by the detection of B cell clonality in benign lesions (11,42), although this issue is controversial (42,43). It is possible that less sensitive, nonmolecular analyses, i.e., the study of κ/λ light chain restriction on minor salivary gland infiltrates in SS, might be of some value to this end. Only the heavily expanded B cell clones would be detected, which might be the ones with the greatest clinical significance. This approach deserves further attention in larger and multicenter studies, since the interpretation or quantification of light chain restriction by immunohistochemical analysis may often be difficult and subjective, especially in borderline cases. Recently proposed in situ hybridization studies might be more reliable (42). However, we stress the role of a careful clinical approach to the patient. In our clinical experience, this has always led to "targeted" tissue biopsies, which allow the diagnosis of SS-associated lymphoma.

In the present study, we have characterized the prelymphomatous stages of B cell lymphoproliferation in SS using multiple and up-to-date molecular analyses of B cell clonality in well-selected tissue lesions from well-characterized patients. The controversial and preliminary conclusions of previous studies can be better evaluated based on the present results. We have provided conclusive evidence that clonal B cell expansion is a frequent event in SS, but may be either oligoclonal or monoclonal, either smaller or larger in size, either fluctuating or established, or either localized or disseminated. Importantly, these different events conceivably imply a different risk of lymphoma progression, though they may all occur under the same pathologic diagnosis of lymphoproliferative lesion. Fully benign lymphoid infiltrates, conversely, appear to be either fully polyclonal or characterized by small oligoclonal B cell expansions. An accurate clinical, histopathologic, and molecular characterization of SS-associated lymphoproliferation in different phases and in different tissues may therefore be crucial in future studies aimed at clarifying the pathobiology of SS-associated lymphomagenesis and the risk of overt malignancy. The clinical approach to borderline cases would then be facilitated.

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