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Larsson, Åke; Bredberg, Anders; Henriksson, Gunnel; Manthorpe, Rolf; Sallmyr, Annahita

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PO Box 117
221 00 Lund
+46 46-222 00 00

Immunohistochemistry of the B-Cell Component in Lower Lip Salivary Glands of Sjögren's Syndrome and Healthy Subjects

Å. Larsson*, A. Bredberg†, G. Henriksson†, R. Manthorpe‡ & A. Sallmyr†

Abstract

*Department of Oral pathology, Centre for Oral Health Sciences, Malmö University; †Department of Medical Microbiology; and ‡Department of Medical Rheumatology, Malmö University Hospital, Lund University, Malmö, Sweden

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Correspondence to: Dr Å. Larsson, Department of Oral Pathology, Centre for Oral Health Sciences, SE-205 06 Malmö, Sweden. E-mail: ake.larsson@od.mah.se

Serial sections of lower lip salivary gland (LSG) biopsies were examined by immunohistochemistry, using a battery of B- and partly T-related antibodies (CD5, CD20, CD21, CD27, CD38, CD45RO, CD79a, Bcl-2 and Bcl-6) in different groups of subjects: healthy controls and clinically verified smoking or nonsmoking cases of primary Sjögren's syndrome (SS). The purpose was to characterize the B-cell pattern of the lymphocytic foci and of the tiny perivascular infiltrates preceding the development of foci. Hyperplastic tonsil was used as stain control. In normal LSG, widely dispersed CD38⁺ and CD79a⁺ as well as some CD5⁺ cells are a normal constituent, with lack of staining with the other antibodies. In SS/LSG, the lymphocytic foci showed staining with all the antibodies, with variable degrees of overlapping or nonoverlapping. In SS/LSG of nonsmokers, CD20⁺ B cells make up a prominent part of the fully developed periductal lymphocytic foci, not overlapping with CD45RO. Also, CD20⁺ B cells did not overlap in the infiltrates with colocalized CD27⁺/CD38⁺ cells. CD20⁺ B cells and CD45RO⁺ T cells also occur as minute infiltrates perivascularly in areas of no foci in SS/LSG as well as in SS smokers lacking the typical foci. Smokers lack foci, but tiny infiltrates express CD20 as well CD45RO. Our findings suggest that CD20⁺ B cells and CD45RO⁺ T cells are early immigrants in the LSG of SS of smokers as well as nonsmokers and that another subgroup of CD27⁺/CD38⁺ B cells gradually mix with the first two to form the characteristic foci in SS/LSG. The simultaneous demonstration of CD20⁺ and CD27⁺ B cells in SS/LSG may constitute a significant diagnostic tool. Further, the findings suggest that the early immigrating lymphocytes may have been primed at a site remote from the glands before arriving via the blood to the gland tissue.

Introduction

Sjögren's syndrome (SS) has long been regarded as a T-lymphocyte-driven disease [1] and a significant amount of data including immunohistochemical findings are related to the T-cell component in the salivary glands of SS. However, chronic activation of B cells may also be a clinically significant component, tentatively contributing to the production of various autoantibodies [2]. Hence, recent data on the overexpression of B-cell-activating factor (BAFF) [3] in the lip minor salivary glands (LSG) of SS cases may be related to excessive survival signals to autoreactive B-cells as suggested by Groom *et al.* [4], who

also found that this BAFF-expressing B-cell component was CD20⁺. This may correspond to mature and/or activated B cells in lymphoid tissue [5]. It has even been suggested that ectopic lymphoid tissue formation is a hallmark of SS [6]. However, in general, B cells have not been excessively analysed by immunohistochemistry in SS.

A diagnostically characteristic LSG finding in SS is focal or autoimmune sialadenitis (AS) [7, 8]. This parameter is included as part of all the different criteria presently applied internationally to diagnose SS [9]. Recently, we found somewhat at our surprise that there was a lower

frequency of AS in the LSG biopsies of smoking SS cases [10]. Smoking seemed to result in a reduced periductal accumulation of lymphocytic cells in the LSG of patients otherwise fulfilling the Copenhagen criteria of SS [11]. This may be due to an anti-inflammatory effect of nicotine [12] likely mediated via a nicotinic acetylcholine receptor [13]. Tentatively, in SS glands, there should still be some lymphocytic changes, but smoking somehow suppressed the development of full-blown foci. Theoretically, analysis of such abortive events should give an insight to the early developmental stages of lymphocytic foci in SS. For natural reasons, such early events are not easily accessible for study in human biopsy material. For example, it has been estimated that the ultimate diagnosis of SS may be made at a time point corresponding to an average of a decade following onset of the SS symptoms [14]. At such a time point, the diagnostically typical focal infiltrates are far beyond the events at which lymphocytic cells initially migrated into the glands. The initial events are poorly understood, eventually resulting in a gradually enlarged focal lymphocytic infiltrate developing over a long period of time in the LSG of SS cases.

It has become increasingly clear that T and B cells may expand oligoclonally in glandular tissue of SS and that B cells are able to undergo a local germinal centre (GC)-like reaction [15], suggestive of ectopic (tertiary) lymphoid tissue. This suggests that B-cell analysis of SS salivary glands may function as a meaningful diagnostic marker adjunct. Using several different B-cell markers, the purpose of the present study was therefore to further examine to what extent an immunohistochemical analysis of lymphocytic B cells in SS/LSG biopsies could function as a useful diagnostic tool. In particular, we made an effort to analyse very small, tiny infiltrates in SS cases which may also be present in localized areas of LSG, expressing fully developed foci in other areas. Such small infiltrates may tentatively correspond to the earliest changes occurring in the development of foci in AS. To further elucidate the meaningfulness of this approach, we included hyperplastic tonsil as a B-cell-rich secondary lymphoid tissue.

Materials and methods

Tissue specimens. Four groups of patient material were used. Formalin fixed, paraffin-embedded lower LSG biopsies were obtained from 10 clinically healthy controls [16] and from 10 clinically verified cases of primary SS retrieved from the files of oral pathology and diagnosed according to the Copenhagen criteria [11]. In addition, 25 biopsies were randomly retrieved from a group of 84 cases previously diagnosed as primary SS but lacking an affirmative focus score, which was found to be clinically correlated with smoking [10]. As a further control group, we performed a restricted analysis of LSG biopsies in 10 consecutive cases, originally biopsied as part of an SS

investigation but not fulfilling the criteria of SS. In retrospect, none of these had serum SSA/SSB. Also, none fulfilled the criteria of AS, i.e. they lacked focal periductal infiltrates, but almost all of these LSG showed tiny lymphocytic infiltrates. Admittedly, the complete smoking history was not available for these 10 cases. Archival paraffin-embedded hyperplastic tonsil specimens used as positive staining controls were from the Pathology Department, Malmö University hospital.

This study was conducted in accordance with the principles defined in the 1964 Declaration of Helsinki, as revised in 1989 by the World Medical Assembly.

Immunohistochemistry. Commercially available antibodies were used (anti-CD3 polyclonal rabbit antihuman, CD5/clone 54/F6, CD20/clone L26, CD21/clone 1F8, CD45RO/clone UCHL1, CD79a/clone JCB 117, Bcl-2/clone 124 and Bcl-6/clone PG-B6p from DAKO; CD27/clone 137B4 and CD38/clone SPC 32 from Novocastra). Efforts were made to produce serial sections, in order to provide for the analysis of the complete immunoprofile of each isolated individual focal aggregate of lymphocytic cells. The sections were deparaffinized to water. For antigen retrieval, the sections were treated by microwave boiling in 0.01 M citrate buffer (pH 6.0) for 10 min. For CD27 and CD38, we performed 10-min microwaving in Tris/EDTA buffer at pH 9. Then, the sections were incubated with primary antibody at optimal dilutions using the DAKO Chem Mate Detection kit K5001 with Tech Mate Horizon automated immunostainer. Staining of controls was performed by the exclusion of the primary antibodies (negative controls) and counterstaining was by Meyers htx.

Results

Normal controls

In lower LSG of normal healthy subjects, CD79a⁺ and CD38⁺ cells were easily demonstrated throughout the tissue, often appearing as small clusters (Fig. 1). In contrast, virtually no cells were reactive to CD20, Bcl-2, Bcl-6, BAFF and CD45RO. Also, single CD27⁺ cells could be demonstrated only with difficulty in the normal glands (sections run in duplicate, at two different occasions). In contrast, CD5⁺ cells were not lacking, with a small number consistently appearing in association with the CD38⁺/CD79a⁺ clusters.

Notably, a small population of CD20⁺ B cells could be detected without difficulty in LSG biopsies of the 10 tentative SS cases, not completely fulfilling the criteria of SS.

Hyperplastic tonsil was used as control tissue, as a background reference of immune cell reactivity [17]. Briefly, T-cell zones clearly stained with CD45RO and CD3, also

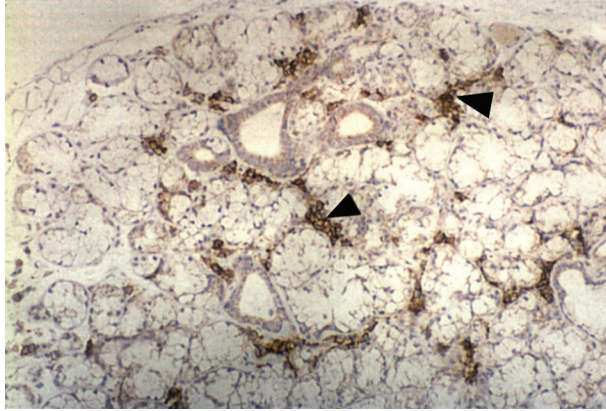


Figure 1 Normal healthy lower lip salivary gland stained with anti CD38. Small groups of cells corresponding to plasma cells (arrowheads) are evenly distributed. Similar results were obtained with anti CD79a, whereas anti CD27 showed only a single positive cell and CD20 was negative. Original magnification $\times 25$.

showing scattered cells in the GC and in the follicular mantle zone (FM). Several CD3/CD45R0-stained cells

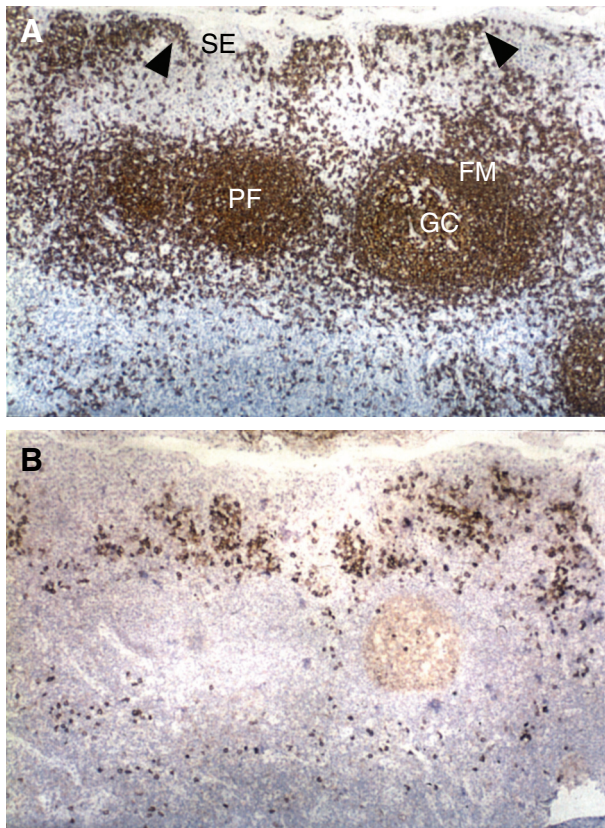


Figure 2 Hyperplastic tonsil, serial sections incubated with anti CD20 and CD38, respectively. Tonsillar crypt at top. (A) Primary follicle (PF), secondary follicle/germinal centre (GC), the follicular mantle zone (FM) and cells in the subepithelial compartment (SE, arrowheads) stain with CD20. (B) The majority of CD38⁺ cells appear in the SE, notably at a level different from CD20 (cf A). There are few or no CD38⁺ cells in PF, GC and FM as labelled in part A. Original magnification $\times 25$.

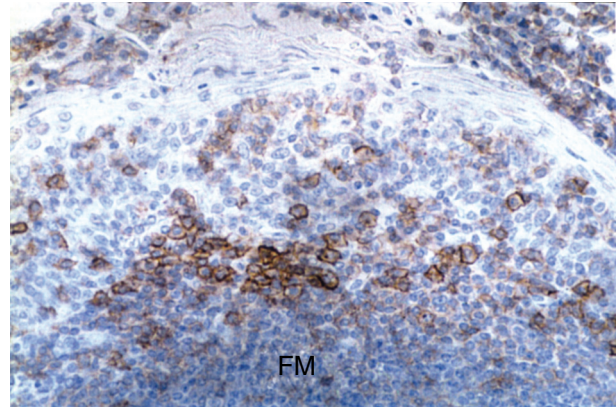
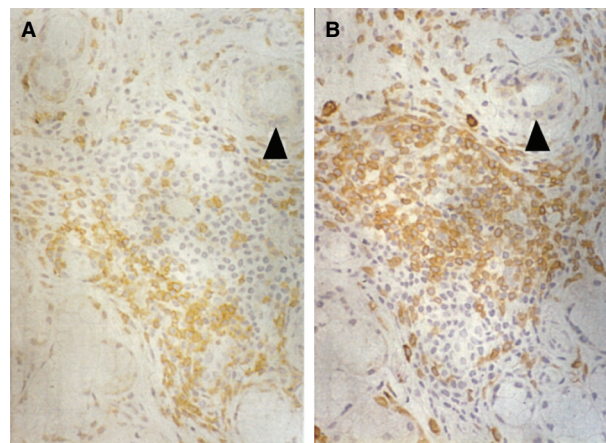
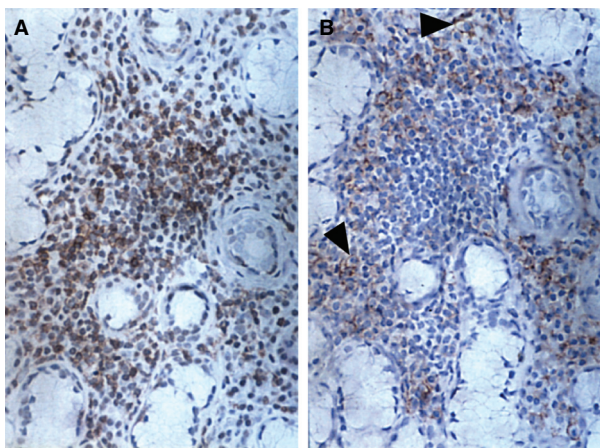


Figure 3 Hyperplastic tonsil at high magnification stained with CD27, crypt epithelium on top (FM, follicular mantle zone). CD27⁺ cells occur at a subepithelial level coinciding with the level of CD38⁺ cells (Fig. 2B). Also note the large size of the CD27⁺ cells, to be compared with CD27⁺ cells in SS/LSG (cf Figs 5B and 8A). Original magnification $\times 50$.

appeared in the extrafollicular/subepithelial compartment (SE). Here, CD5-stained cells were frequent, showing a tendency to accumulate at the epithelial interface. Scattered CD5-stained cells appeared in the centroblastic part of the GC but more frequent in the centrocyclic. The most prominent CD5 staining was in the T cell zones, whereas the majority of cells in the primary follicles and FM lacked CD5 staining. Bcl-2 staining was prominent in the FM, with scattered cells in the GC but only few stained cells extrafollicularly including the SE [18]. Bcl-6 staining was localized to GC only and this was also the case with CD21. CD20/27/38/79a staining overlapped to some extent. In summary, primary follicles stained heavily with CD20 and CD79a, weakly with CD27 and negatively with CD38 (Fig. 2A,B). The FM zone cells were evenly and strongly stained with CD20 and weakly with CD79a whereas



Figures 4 Serial sections of SS/LSG (arrowhead, salivary duct), illustrating lymphocytic focus with lack of overlapping between CD45R0⁺ (A) and CD79a (B). Original magnification $\times 50$.

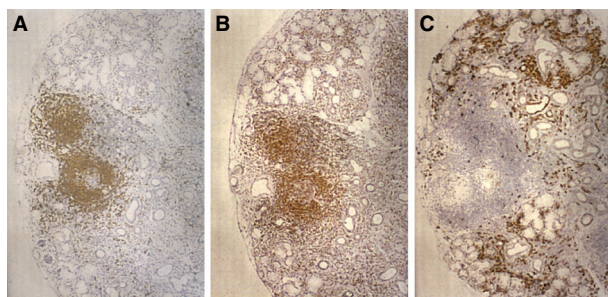


Figures 5 Serial sections of SS/LSG, showing lymphocytic focus with lack of overlapping between CD45R0⁺ (A) and CD27 (B). Note large size of plasma membrane stained CD27⁺ cells at periphery of focus (arrowheads, cf tonsil Fig. 3). Original magnification ×50.

scattered and essentially no FM cells stained with CD27 and CD38, respectively. Strongly stained cells were found in the subepithelial (SE) 'marginal zone (MZ)-like' zone [19, 20]. In serial sections, CD20⁺ and CD38⁺ cells tended to appear at different levels of this zone, with CD20⁺ closely associated with the surface epithelium and CD38⁺ at a lower level (Fig. 2A,B), the latter coinciding with the majority of CD27⁺ cells (Fig. 3). The SE zone also showed strongly as well as weakly CD79a-stained cells.

Lower LSG of SS cases

In LSG fulfilling the criteria of autoimmune/focal sialadenitis/AS, the lymphocytic foci (a focus defined as at least 50 periductal cells, cf 7) expressed CD45R0, not overlapping with a CD20⁺/CD79a⁺ cell population (Fig. 4A,B), nor with CD27 (Fig. 5A,B). CD20 was consistently expressed in the foci (Fig. 6A), and it overlapped with Bcl-2 (Fig. 6B), but not with CD38 (Fig. 6C). Occasionally, but not always, such foci showed Bcl-6⁺ cells appearing as focal



Figures 6 Serial sections of SS/LSG showing two foci with overlap of staining between CD20 (A) and Bcl-2 (B) but not with CD38 (C). Original magnification ×25.

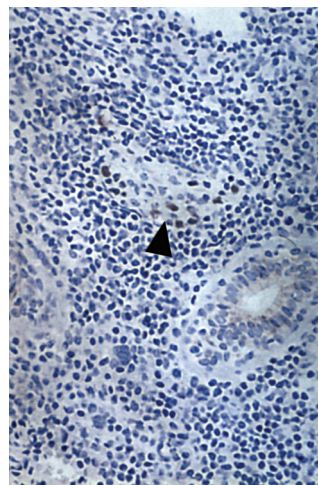
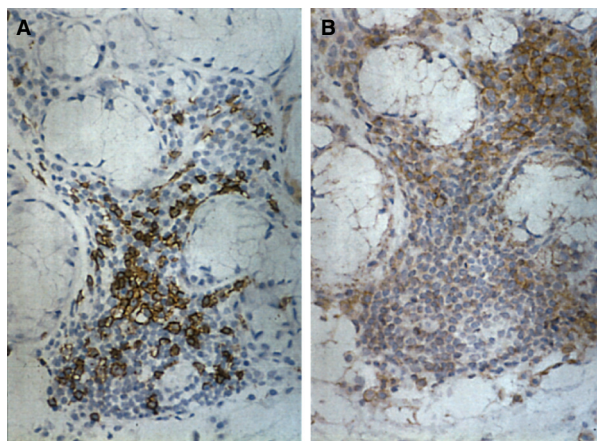


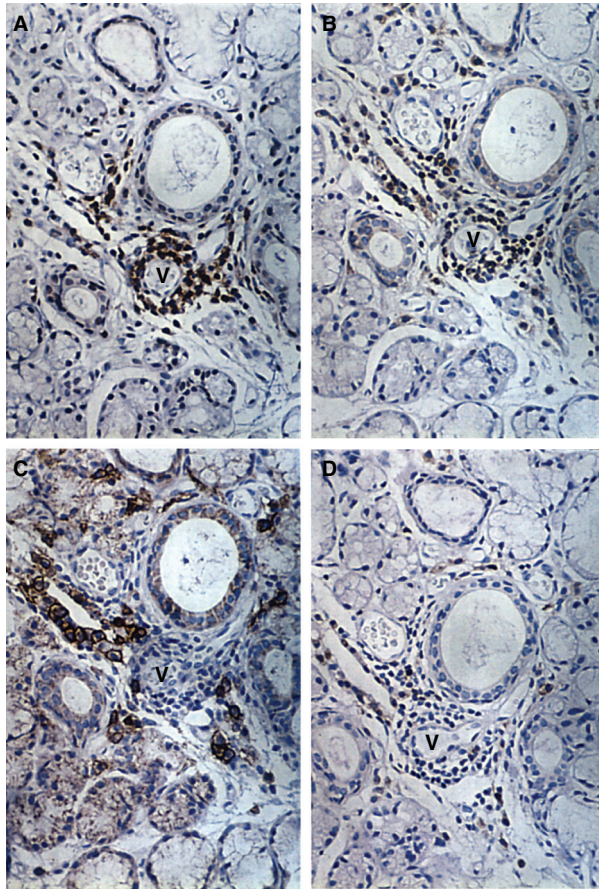
Figure 7 High magnification of lymphocytic focus in SS/LSG stained with anti Bcl-6. A small group of Bcl-6⁺ cells (arrowhead) reflects ongoing development of a secondary follicle/germinal centre (see text for details). Original magnification ×50.

aggregates (Fig. 7) interpreted as early phases of secondary follicle/GC formation, reflecting the Bcl-6 stain pattern in the hyperplastic tonsil (above). The CD20⁺ foci expressing Bcl-2 showed peripherally located CD38⁺ as depicted in Fig. 6A, C, reminiscent of primary follicles in lymphoid tissue/tonsil. CD20 did not overlap with CD27 (Fig. 8A,B). We were able to confirm previously reported BAFF reactivity as well as BCA-1 associated with the CD20⁺ infiltrates [3, 21] (not illustrated).

The LSG biopsies of the SS/smokers lacked lymphocytic foci. These glands showed a CD38 and CD79a pattern not much different from healthy controls. However, in addition, they showed small perivascular, but not periductal, infiltrates. Such infiltrates were virtually absent in normal healthy controls, but such small as well as inter-

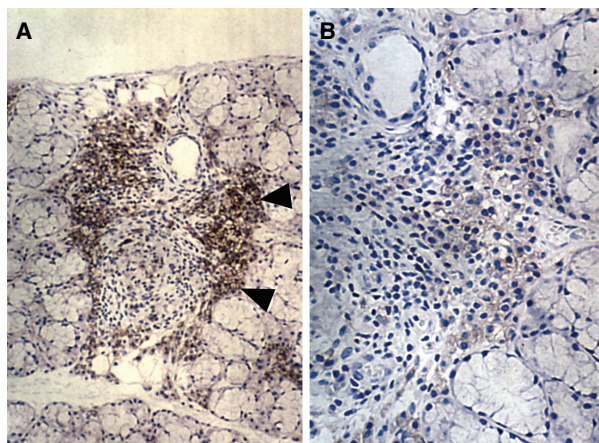


Figures 8 Serial sections of SS/LSG showing CD20⁺ cells within a small focal infiltrate (A) with no overlapping with CD27 (B). Original magnification ×50.

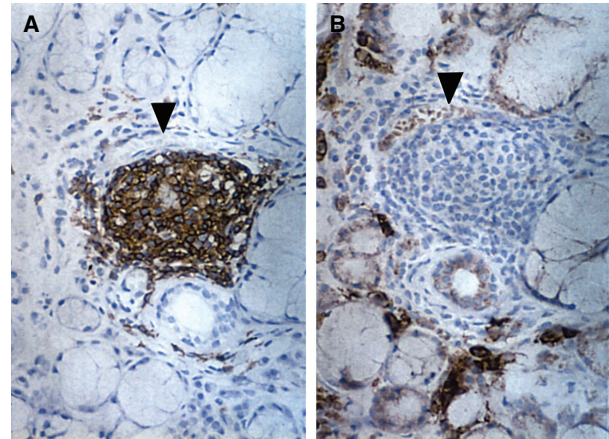


Figures 9 Serial sections of SS/LSG showing a tiny perivascular lymphocytic infiltrate (v, vessel) staining positively with CD45R0 (A) and Bcl-2 (B) but negatively with CD38 (C) and CD79a (D). Original magnification $\times 50$.

mediate-sized infiltrates could also be demonstrated in LSG of nonsmokers in areas remote from the typical



Figures 10 Serial sections of SS/LSG showing a lymphocytic focus stained with CD38 and CD27, respectively. Note peripherally located CD38⁺ cells, cf Fig. 6C. The CD38⁺ cells at arrowhead in (A) overlap with CD27⁺ cells depicted at higher magnification in (B). Original magnification $\times 25$ and $\times 50$, respectively.



Figures 11 Serial sections of SS/LSG showing a small localized CD20⁺ infiltrate (A) not overlapping with CD38 (B), cf Fig. 6A, C. Blood vessel at arrowhead. Original magnification $\times 50$.

foci. In SS/LSG, lymphocytic cells in small perivascular infiltrates invariably expressed CD45R0, which overlapped with Bcl-2 (Fig. 9A,B). These cells did not overlap with CD20⁺, CD79a⁺ and CD38⁺ cells (Fig. 9C,D). Hence, Bcl-2⁺ cells overlapped with tiny perivascular CD45R0⁺ cells but in this situation, the Bcl-2⁺ cells did not clearly overlap with CD20⁺ cells, apparently in contrast to larger foci (above). In addition, the CD38-stained cells associated with the foci overlapped with CD27 (Fig. 10A,B), which was not the case in normal healthy glands where CD38⁺ cells were unaccompanied by CD27⁺ cells (above). Also in the SS/LSG, the CD38⁺/CD27⁺ cells did not overlap with the CD20⁺ cells (Fig. 11A,B).

We performed an incomplete analysis of CD5-stained cells, which were readily detected in foci but not clearly seen in the very tiniest perivascular infiltrates. They did not overlap with CD27 (Fig. 12A,B), indirectly suggesting

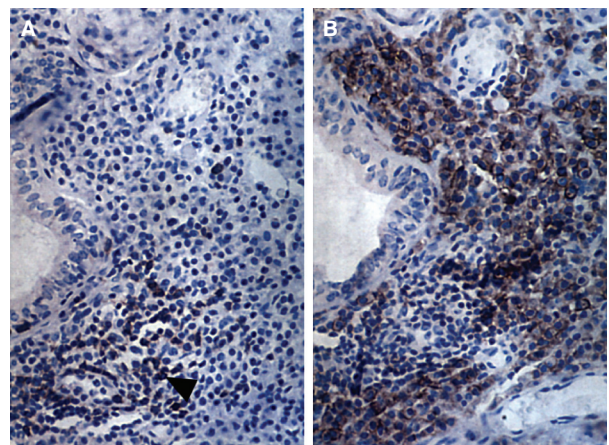


Figure 12 Serial sections of SS/LSG showing part of a lymphocytic focus showing a localized aggregate of CD5⁺ cells (A, arrowhead) not overlapping with CD27⁺ cells (B). Original magnification $\times 50$.

that the CD5-stained cells do not belong to the CD27⁺/CD38⁺ cells, see above.

Discussion

In the present study, an effort has been made to map the B-cell pattern in lower LSG biopsies of SS. We found evidence of three different lymphocytic populations, one T cell, CD45R0⁺, and two B cell, CD20⁺ and CD38⁺/CD27⁺. The exact interrelationship between the different cell populations remains to be elucidated. In SS/LSG, large, sometimes even confluent, foci are mixed with different lymphocytic cells. Admittedly, such study material is not homogeneous, meaning that the exact developmental stage is not known neither within each individual lymphocytic infiltrate nor between the different patients. Also, the complexity of such foci makes it virtually impossible to gain insight into the initial events of cell infiltration. Nevertheless, a specific aim of our study has been to try to identify small, tentatively early perivascular lymphocytic infiltrates in LSG, which could correspond to events preceding the development of the typical periductal foci. For that purpose, we also included LSG of SS/smokers. We reasoned that as these SS cases show only limited tendency to develop lymphocytic foci [10], they may provide an opportunity to identify such early infiltrates. Additionally, it may be possible theoretically by serial sectioning to characterize a single very small lymphocytic LSG infiltrate with several different antibodies. In practice, however, there is a limit to how many serial sections that can be taken still containing that same minute infiltrate. In addition, the whole set of different antibodies must work optimally by the time of incubation. With the presently used antibodies to Bcl-2, Bcl-6, CD5, CD20, CD27, CD38, CD45R0 and CD79a, it may seldom be possible to secure a complete set of serial sections containing a localized small infiltrate. It should again be emphasized that the time course of development of all the separate lymphocytic foci within one and the same LSG biopsy may differ somewhat in detail from each other. Even though the findings we have reported were present in all of the SS/LSG, the present results should be interpreted with some caution, being tentative with respect to the earliest events of lymphocytic infiltration.

Hyperplastic tonsil was included as a control tissue, partly in order to analyse to what extent immunohistochemical analysis of B-cell markers may correlate in a meaningful way with current studies on isolated tonsil B cells [19, 20, 22]. This could possibly increase our understanding about the mechanisms of B-cell infiltration in SS/LSG and possibly offer additional diagnostic avenues, which presently rely somewhat stereotypically on counting of lymphocytic foci in routinely stained sections of LSG [7, 23].

There may be seven B-cell subpopulations in tonsil [5]. Our restricted effort showed that the staining pattern in tonsil was essentially in accordance with the observations made in different B-cell compartments by Marafioti *et al.* [24], in addition to the fact that some of our antibodies also stain T cells (e.g. CD27) [25]. Staining with CD20/CD27/CD38/CD79a antibodies showed some degree of overlapping but also distinctly separate patterns of staining. Primary follicles/PF as well as the FM zones of GC stained with CD20 and CD79a, but sparsely with CD27 and CD38. As expected, CD38 clearly stained extrafollicular cells as well as some GC cells. In addition, the finding in lower LSG that a prominent CD38⁺ population of cells did not overlap with CD27 in healthy control glands but overlapped in the SS cases suggests that terminally differentiated CD38⁺ plasma cells do not stain with CD27. In comparison, Marafioti *et al.* [24] suggested that CD38⁺ plasma cells also express CD27, similar to the present CD38⁺/CD27⁺ B cells in the SS/LSG.

With CD20, the present study showed that the lymphocytic infiltrates in the SS/LSG including the smallest perivascular had a prominent CD20⁺ component, with CD20⁺ cells virtually lacking in healthy glands. This is in accordance with previous data [4]. Notably, the infiltrates also expressed a CD20 nonoverlapping population of CD38⁺ cells, which clearly overlapped with CD27, lacking in normal glands. Critically, the CD38 as well as the CD27 antibody both react with B as well as with activated T cells, at least in flow cytometry systems. However, the present immunohistochemical findings suggest that as the CD38/CD27-stained cells did not overlap with the CD45R0 T-cell-specific marker, the former should correspond to B cells. One conclusion that can be made is therefore that there are at least three nonoverlapping populations of lymphocytic cells in the LSG infiltrates of SS, namely CD45R0⁺/Bcl-2⁺ T cells and two separate populations of B cells, one CD20⁺ (and also CD5⁺?) and the other CD38⁺/CD27⁺. Interestingly, this pattern of staining is reminiscent of the CD20/CD27/CD38 staining which we observed in the SE zone of hyperplastic tonsil, with CD20 staining being separate from CD27/CD38.

CD20, as previously demonstrated in the SS/LSG lymphocytic infiltrates [4], is expressed in B cells from the stage of pre-B and immature into mature cells as well as activated B, but not plasma, cells [26]. Accordingly, we found that all the different compartments in primary and secondary tonsil follicles demonstrate CD20⁺ B cells. Although the exact function of the CD20 gene is not clear [27], there is much current interest in the potential therapeutic efficiency of chimeric monoclonal anti CD20 for the treatment of not only lymphoma [28] but also autoimmune disorders involving B cells [29]. As also confirmed in the present study, the CD20⁺ infiltrates in SS

glands have been found to express BAFF, which does not affect proliferation, but is required for the persistence of mature B cells [3]. An intriguing question is if these CD20⁺ B-cell infiltrates correspond to pre- or post-GC type B cells. The former would correspond to mature peripheral blood-derived B cells, not yet activated via a lymphoid follicular/GC program, whereas the latter have by definition passed such a program. The accumulated CD20⁺ infiltrates correspond morphologically to primary follicles. Even though single SS/LSG cases may show Bcl-6 staining, suggesting transformation to secondary follicles/GC [30], our experience is that most SS/LSG cases show little or no such tendency. Thus, Bcl-6 staining in order to detect evidence of GC formation may not represent an improved diagnostic tool in SS/LSG.

The present findings in the SS/LSG infiltrates demonstrated that CD38⁺ cells do not overlap with CD20 and that CD20 staining does not overlap with CD27, which in turn does not stain plasma cells. All of these three markers stained B cells in the SE zone of the tonsil although at different levels, confirming different subtypes of B cells [19]. Ongoing studies of isolated human tonsil B cells suggest the possible existence of extrasplenic MZ B cells likely corresponding to a subpopulation of B cells in the extrafollicular SE compartment of the tonsil [19, 20, 22]. The majority of such MZ-like B cells in the SE zone have mutated IgV genes consistent with memory B cells which have passed a GC program. Falini *et al.* [22] found that CD27⁺ B cells isolated from the tonsil SE showed either mutated or unmutated IgV genes, suggestive of two different memory-type B-cell populations in the SE. Tentatively, the presently demonstrated CD27⁺/CD38⁺ LSG/SS B cells may correspond to a population of MZ-like CD27⁺/CD38⁺ B cells described in the tonsil SE zone by Dono *et al.* [19]. Notably, Groom *et al.* [4] also suggested that MZ-like B cells may participate in the SS/LSG infiltrates.

In tonsil, the majority of CD5⁺ cells has the phenotype of FM B cells [20]. However, in the present tonsil study, the FM did not show the most prominent CD5 staining, which was seen in the T-cell zones. There is a CD5-positive/CD38-negative fraction of MZ-like B cells in the tonsil [20, 31], which may correspond to some of the presently observed CD5⁺ cells in that area. They may also express CD27 [20], but the CD5⁺ cells in the present SS/LSG did not overlap with CD27. In general, as more than 95% of human T cells also express the CD5 glycoprotein detected by the presently used CD5 antibody and as also suggested by the present prominent tonsil T-zone CD5 staining, the use of CD5 marker antibody may present unsurmountable obstacles in immunohistochemistry of secondary lymphoid tissue B cells. This may also be the case with the T- and B-cell-rich infiltrates in SS/LSG. In contrast to ambient flow cytometry data on CD5 in the literature, this may partly explain the paucity

of immunohistochemical reports about CD5 in SS/LSG, in spite of the well-known fact that SS cases may show high levels of circulating CD5⁺ lymphocytes [32]. Notably, we readily detected a small number of CD5-stained cells in the SS/LSG, confirming previous isolated immunohistochemical data [33]. The fact that the present SS/LSG CD5⁺ infiltrates did not overlap with the CD27⁺ cells indirectly suggests that neither a correlation with T cells (CD45R0) nor with CD20⁺ B cells can be excluded. On the other hand, the fact that CD5⁺ cells can also be demonstrated in normal glands makes their significance in SS/LSG uncertain.

Of the currently used B markers, CD79a may be the least 'specific'. It detects a polypeptide that appears early in B-cell maturation persisting until the plasma cell stage, then giving a strong staining [34]. However, it may still be rewarding to review our CD79a results. Arguably, the mixture of weak and strong CD79a-stained cells in the SE zone of the tonsil could correspond to the presently demonstrated CD20⁺ and CD38⁺ SE cell population, respectively, given the fact that a weak CD79a staining overlaps with a strong CD20 in the FM and that CD79a and CD38 both give a strong staining to plasma cells. Our findings in the SS/LSG foci of a weakly CD79a-stained population admixed with the strong CD20⁺ cells indirectly suggest that these B cells could correspond to a mature, peripheral blood derived naive B cell of a primary follicle or FM type rather than to MZ-like B cells in the SE. This may seem to be further supported by the fact that the CD20⁺ foci in SS/LSG as well as the FM were essentially CD27 negative, raising the suspicion that the CD38⁺/CD27⁺ cells could be MZ-like B cells.

Smoking may somehow interfere with migration of lymphocytes into LSG in SS cases [10]. Such LSG biopsies may not fulfill the complete criteria of focal or autoimmune sialadenitis/AS. However, they are not avoid of lymphocytic infiltrates. In the present study, one of the objectives was therefore to look for signs of abortive AS in LSG of SS smokers with nonconfirmed AS. CD20 seemed like a particularly promising candidate marker, due to its absence in healthy glands and its prominent staining in AS. Indeed, LSG of SS smokers demonstrated tiny CD20⁺ infiltrates, suggesting that they could correspond to an early initiating phase of AS, thus giving further support to the previous findings that smoking may interfere with but not completely stop infiltration of lymphocytes in the SS LSG. Also, we found that CD20⁺ B cells may be present as tiny infiltrates in LSG of cases not fulfilling the complete criteria of SS. Even though the smoking history of these cases was unclear, the findings indirectly suggest that the presence of CD20⁺ B cells may reflect a slowly ongoing pathologic process lacking in normal healthy glands. However, our data do not permit the conclusion, that CD20⁺ B-cell infiltration in LSG may be a diagnostic event exclusively related to SS.

The CD27 findings may be of particular interest. Normally, in secondary lymphoid tissue, the follicular B-cell program results in differentiation into plasma cells or memory CD27⁺ B cells [35]. Normal healthy LSG virtually lack CD27⁺ cells, in contrast to the SS glands of smokers and nonsmokers. This is similar to previous findings where Hansen *et al.* [36] suggested that the accumulation of memory CD27⁺ B cells in the SS glands may correlate with their reduction in peripheral blood, as also supported by other data [37]. Topographically, the present findings demonstrate that CD27⁺ B cells accumulate in close association with CD38⁺ cells, but they do not overlap with the CD20⁺ cells. If CD27⁺ memory B-cell accumulation in SS glands may be a result of an increased influx of peripheral blood CD27⁺ cells, then peripheral blood CD27⁺ B cells must derive from a follicular differentiation program at some other remote site of the body before arriving at the SS glands. In addition, the CD27⁺ population does not overlap with CD45R0. This suggests that the CD27⁺ population is predominated by memory type B cells and that the CD45R0⁺ cells may correspond to memory type T cells [38]. Being nonsignificant in normal tissue, the CD27-stained cells which appeared in significant amounts in the SS glands can hardly correspond to a pool of long-lived quiescent memory B cells similar to the spleen pool [39], but must reflect part of the pathologic process in the SS glands, as also suggested by Hansen *et al.* [36]. Also, the CD27⁺/CD38⁺ B cells may seem not to correspond to unmutated CD27⁺/CD38⁻ B cells, which occur in the SE compartment of the tonsil suggested to be the result of mature B cells exposed to local antigenic stimulation [31]. The CD27⁺/CD38⁺ cells may correspond to a plasma cell precursor subset, similar to what was recently demonstrated to be expanded in SLE [40, 41]. The fact that these cells unexpectedly did not overlap with CD20 could tentatively be explained by downregulation of CD20 as a consequence of mechanisms of T–B collaboration [42]. Our findings suggest that CD27⁺ memory B cells in SS/LSG have migrated to the salivary glands following their generation in a remote secondary lymphoid organ [15].

To summarize, provided that lymphocytic sialadenitis of minor glands are representative of SS equal to the classical myoepithelial sialadenitis of major glands [6], the present findings are suggestive of specific B-cell patterns. Tentatively, the combined demonstration of CD20⁺ and CD27⁺ cells may represent an improved diagnostic tool in SS/LSG. The very earliest tiny lymphocytic infiltrates observed in SS/LSG are perivascular CD20⁺/CD79a⁺/Bcl-2⁻ B cells and CD45R0⁺/Bcl-2⁺ T cells, but it is not possible at present to tell exactly which is the first. It is difficult to comprehend how these T cells could turn into a memory or primed phenotype (CD45R0⁺) immediately following migration through the endothelial barrier. A

report of experimentally induced infiltration of salivary and lacrimal glands by memory type T cells [43] gives support to the interpretation that they were already primed at arrival, with an ill-defined antigenic specificity [44]. The early influx of a B-cell population may also be explained by properties of these cells achieved at some remote site of the body, as a high fraction of activated peripheral blood naive B cells have been reported for SS patients [45]. It may be similar to migration of virus-activated B cells into peripheral nonlymphoid tissue [46]. Recruitment of primed T and B cells to the gland does not exclude that local vascular and/or epithelial factors may attract these lymphocytes [47]. It seems that the CD27⁺/CD38⁺-differentiated B cells become added to the infiltrates at a later time point, resulting in two distinct B-cell populations in the proper foci, raising a suspicion that these CD27⁺/CD38⁺ B cells may correspond to CD27⁺/CD38⁺/CD20⁻ plasma cell precursors similar to those found to be expanded in SLE [41]. In addition to possible immigration from peripheral blood of CD27⁺ B cells, it is tempting to speculate that some CD27⁺ B cells may be generated *in situ*, by mechanisms of T–B collaboration involving the CD40L–CD40 system reported to be upregulated among the infiltrating mononuclear cells in SS/LSG [48]. This is supported by observations of local tissue CD40L–CD40-mediated B-cell differentiation including class switch recombination in the absence of antigen [49] and in the absence of GC structures [50, 51] in the gut.

As a final conclusion, our findings emphasize the hypothesis of an enhanced stress response as an early event in the pathogenesis of SS, leading to stress-induced hyperactivity among the immune cells [52] and to the hallmark clinical symptoms due to the salivary gland being a locus minoris resistentiae to stress signals [53–55].

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