

Attenuated Apoptosis of B Cell Activating Factor–Expressing Cells in Primary Sjögren’s Syndrome

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SUMMARY: B cell activating factor (BAFF) is known to be a powerful regulator of B-cell differentiation and proliferation. The aim of this study was to assess the incidence of apoptosis among BAFF-expressing cells in Sjögren’s syndrome (SS) salivary gland tissue. We performed double stainings of BAFF together with one of the markers, CD21, CD68, CD40, Fas, Bcl-2 or Bax, and monitored apoptosis among BAFF expressing cells by using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling method. A significantly lower level of apoptosis among the BAFF-expressing cells was detected in salivary glands from patients with SS compared with controls ($p = 0.03$). Furthermore, no difference in the coexpression of Fas or CD40 together with BAFF was detected between patients and controls. Coexpression of the pro apoptotic molecule Bax together with BAFF was nonsignificantly decreased in patients with SS compared with controls. Our results suggest that a reduced level of apoptosis among BAFF-expressing cells might lead to longer-existing BAFF expression within these cells and thereby maintain signaling for tissue-infiltrating B cells to proliferate and mature. (*Lab Invest* 2003, 83:357–365).

Sjögren’s syndrome (SS) is a chronic, slowly progressive, systemic autoimmune disease, which predominantly affects middle-aged women, although it can be seen in patients of all ages, including children (Jonsson et al, 2002). It is characterized by lymphocytic infiltration and destruction of the exocrine glands, resulting in xerostomia and keratoconjunctivitis sicca and the presence of other exocrinopathic symptoms (Jonsson et al, 2002). SS can develop alone (primary SS) or in association with other autoimmune diseases (secondary SS) such as rheumatoid arthritis, systemic lupus erythematosus, polymyositis, systemic sclerosis, or thyroiditis (Jonsson and Brokstad, 2001; Manoussakis et al, 1998).

B-cell activation is a consistent immunoregulatory abnormality in SS, in which the B cells constitute roughly 20% of the infiltrating cell population in exocrine glands. IgG is the predominant isotype expressed by the infiltrating B cells, in contrast to IgA, which dominates in normal salivary glands (Halse et al, 1999). Furthermore, several autoantibodies have been reported in both primary and secondary SS, reflecting both B-cell activation and a loss of immune tolerance in the B- and T-cell compartments (Jonsson et al, 2001b). During the past few years, there have been significant advances in defining the fine specificity of

these Abs and characterizing their target autoantigens. In some cases, the autoantibodies are correlated with the extent and severity of disease in SS and are potentially involved in the pathogenic process of the autoimmune exocrinopathy (Jonsson et al, 2001a).

The TNF superfamily ligand BAFF (B cell activating factor) also known as TALL-1, THANK, BlyS, and zTNF4, enhances B-cell survival in vitro and has emerged as a key regulator of the peripheral B-cell population in vivo (Batten et al, 2000; Mukhopadhyay et al, 1999; Schneider et al, 2001; Thompson et al, 2000). BAFF binds to B cells and costimulates their growth in vitro. Mice transgenic (Tg) for BAFF have vastly increased numbers of mature B and effector T cells and develop autoimmune-like manifestations such as the presence of high levels of rheumatoid factor, circulating immune complexes, anti-DNA autoantibodies, and Ig deposition in the kidneys. This phenotype is reminiscent of certain human autoimmune disorders and suggests that dysregulation of BAFF expression may be a critical element in the chain of events leading to autoimmunity (Jiang et al, 2001; Mackay et al, 1999).

A recent study showed increased levels of circulating BAFF in patients with SS and up-regulation of BAFF expression in inflamed salivary glands (Groom et al, 2002). The presence of a marginal zone–like phenotype of the infiltrating B cells within the salivary glands of BAFF Tg mice suggests that cells of this compartment potentially participate in tissue damage in SS (Groom et al, 2002). Furthermore, BAFF enhances both T cell-independent and T cell-dependent humoral immune responses, which imply a role for BAFF in the maintenance of the B-cell repertoire. The ability of BAFF to increase B-cell survival suggests

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that attenuation of apoptosis underlies BAFF enhancement of polyclonal autoimmunity (Do et al, 2000).

The purpose of this study was to describe the localization and quantity of BAFF-expressing cells in relation to tissue-infiltrating macrophages, B cells, and various pro- and antiapoptotic markers in the salivary glands of patients with primary SS compared with controls. Here, we describe a significantly lower level of apoptosis among BAFF⁺ cells derived from primary Sjögren's syndrome-salivary gland tissue (SS-SG) compared with controls. Our results suggest a prolonged existence of BAFF-expressing cells within salivary gland-infiltrating cells and thereby a possible maintenance of a signal for tissue-infiltrating B cells to proliferate and mature into in situ Ab-producing cells taking part in the autoimmune machinery. Indeed, these findings can also be explained by an increased clearance of apoptotic cells in SS-SG, leading to reduced levels of BAFF⁺ apoptotic cells.

Results

Specificity of anti-BAFF mAbs for BAFF

To determine the specificity of anti-BAFF mAbs used in this study, we performed an assay in which addition of increasing amounts of recombinant soluble BAFF protein together with anti-BAFF mAbs inhibited tissue staining in a dose-dependent manner (Fig. 1). This shows that anti-BAFF Abs are specific for BAFF. The Ab we used in this study recognizes the stalk region present in the membrane-bound form of hBAFF (amino acid 83–285), and image analysis showed a ring-shaped membrane-specific staining pattern with anti-BAFF, proving that the Ab is targeted against the membrane-bound form of BAFF.

Localization of BAFF⁺ Cells and Different Apoptotic Markers in the Salivary Gland

Both regular immunohistochemical staining and immunofluorescent labeling were performed to localize BAFF-expressing cells. The identification of positive cells was performed by merging fluorescent images

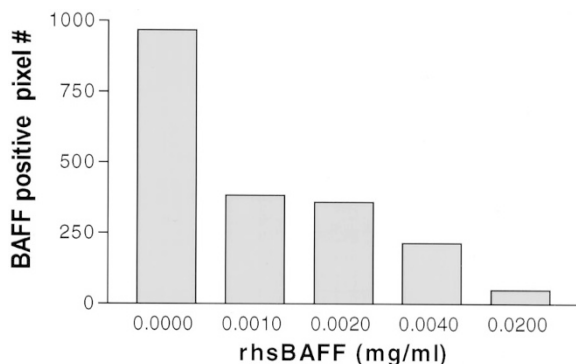


Figure 1.

Different concentrations of recombinant human soluble BAFF (*rBsBAFF*; 0.00–0.02 mg/ml), with the simultaneous application of anti-human BAFF Ab, inhibited tissue staining in a dose-dependent manner.

with hematoxylin-stained light microscope images (Fig. 2).

Regular immunohistochemical staining showed a prominent expression of BAFF on tissue-infiltrating cells, as well as on ductal and acinar epithelial cells (Fig. 3, A, C, and D). Furthermore, ductal epithelial cells showed a more intensive staining for BAFF than the acinar epithelium (confirmed by quantitation of pixels and pixel luminosity values; data not shown). These findings were also confirmed by immunofluorescent staining.

The immunofluorescent images of primary SS-SG infiltrates showed ring-shaped, membrane-bound staining, which confirms that the staining was targeted against membrane-bound BAFF (Fig. 3E). CD21 (B-cell marker) was expressed on infiltrating mononuclear cells in SS and sparsely among interstitial cells in controls. The majority of macrophage (CD68) staining appeared on tissue-infiltrating cells, whereas some staining was also detected on ductal and acinar epithelial cells. Immunohistochemical and immunofluorescent stainings of CD40, Fas, Bcl-2, and Bax were also performed. Briefly, CD40 and Bcl-2 occurred abundantly in SS-SG infiltrating mononuclear cells, whereas Fas and Bax appeared mostly in acinar and ductal epithelial cells. In every case, the two different staining methods gave the same results. In situ detection of programmed cell death by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) staining revealed that apoptosis appeared most frequently among infiltrating cells (Fig. 3E), whereas ductal and acinar epithelial cells were less frequently TUNEL positive.

Quantitative Analysis of BAFF, CD40, Bcl-2, Bax, Fas, and Apoptosis In Situ

Comparative data were obtained by quantitative evaluation of salivary gland histologic examination in eight patients with primary SS and eight controls. To estimate the total BAFF⁺ pixel numbers and luminosity intensities, 120 primary SS and 120 control group fluorescent microscope images were analyzed and compared. The total number of BAFF⁺ pixels was nonsignificantly increased in the primary SS group compared with controls (C) (SSmean = 1738 pixels vs. Cmean = 1387 pixels, $p = 0.327$). Similarly, the CD21 positive pixel-number (SSmean = 1409 pixels vs. Cmean = 1147 pixels, $p = 0.326$) and the mean luminosity intensity (SSmean = 108.2 vs. Cmean = 97.3, $p = 0.362$) were also nonsignificantly increased in the primary SS group compared with controls. Total Bax (SSmean = 1203 pixels vs. Cmean = 1138 pixels, $p = 0.786$), CD40 (SSmean = 1286 pixels vs. Cmean = 1220 pixels, $p = 0.817$), and Bcl-2 pixel levels (SSmean = 953.6 pixels vs. Cmean = 1040 pixels, $p = 0.698$) were comparable between primary SS patients and controls.

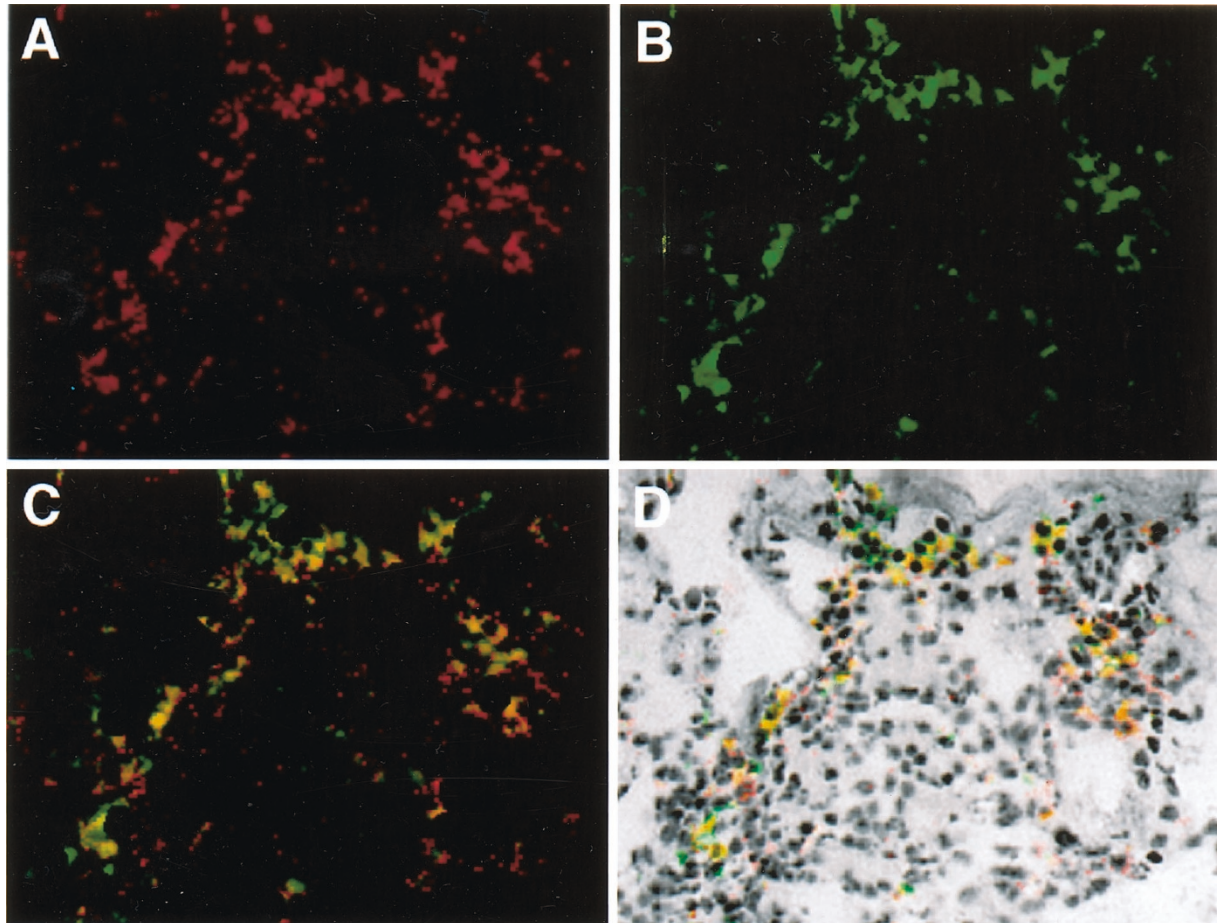


Figure 2.

Identification of fluorescent pixel localizations. A, Red spectral emission signals represent BAFF staining. B, Green spectral emission signals represent Bcl-2 staining. C, Overlaid image of A and B. Identical red and green pixels emission signals appear in the yellow spectrum. D, Merged image (C) overlaid on identical light microscopy picture of a Sjögren's syndrome salivary gland tissue (SS-SG) to identify the localization of fluorescent single (red and green) and double-positive (yellow) signals. D, Most of the red, green and yellow signals are localized on tissue-infiltrating cells. Quantification of single- and double-positive pixel numbers and pixel luminosities were determined by Photoshop graphics software.

To describe the coexpression/localization of different apoptotic markers and BAFF and to quantify the frequency of apoptosis among BAFF⁺ cells, we performed double-fluorescence labeling and we quantified single- or double-positive pixel numbers in addition to luminosity intensity values. Interestingly, a significantly lower level of TUNEL-positive pixels were detected in primary SS-SG among the BAFF⁺ cells as compared with controls ($p = 0.03$) (Fig. 4). Furthermore, lower Bax and BAFF double-positive pixel counts were detected in patients compared with controls, although this did not reach a statistical significance (SSmean = 523.3 pixels vs. Cmean = 720.7 pixels, $p = 0.253$). The CD21-BAFF colocalization and CD40-BAFF coexpression was found in comparable amounts in patients and controls (not shown). We also found similar levels of double-positive pixel numbers for CD95-BAFF and Bcl-2-BAFF, showing that the coexpression of these markers were comparable among primary SS and controls (not shown). No significant differences in mean luminosity intensity values between primary SS patients and control groups for any of the markers were found.

Discussion

It has been proposed that imbalance in BAFF production could be a major factor contributing to the development of SS through the recruitment and activation of a specific and potentially pathogenic subpopulation of B cells that targets exocrine glands (Groom et al, 2002). In this study, we further investigate BAFF⁺ cells in SS-SG in relation to apoptosis. We describe the localization of BAFF expression, and we quantify BAFF expression with pro- and antiapoptotic molecules in SS-SG.

Our study revealed that BAFF appeared abundantly among infiltrating cells and, to a lesser extent, among ductal and acinar epithelial cells. In our experiments we used an anti-BAFF mAb, which is targeted against the stalk region of the membrane-bound form of BAFF. The specificity of the Ab was evaluated by using a dose-dependent inhibition assay with recombinant human BAFF protein. The BAFF staining pattern showed a pericellular, ring-shaped form that further suggests that the membrane-bound form of BAFF has been investigated in this study. BAFF⁺ pixel

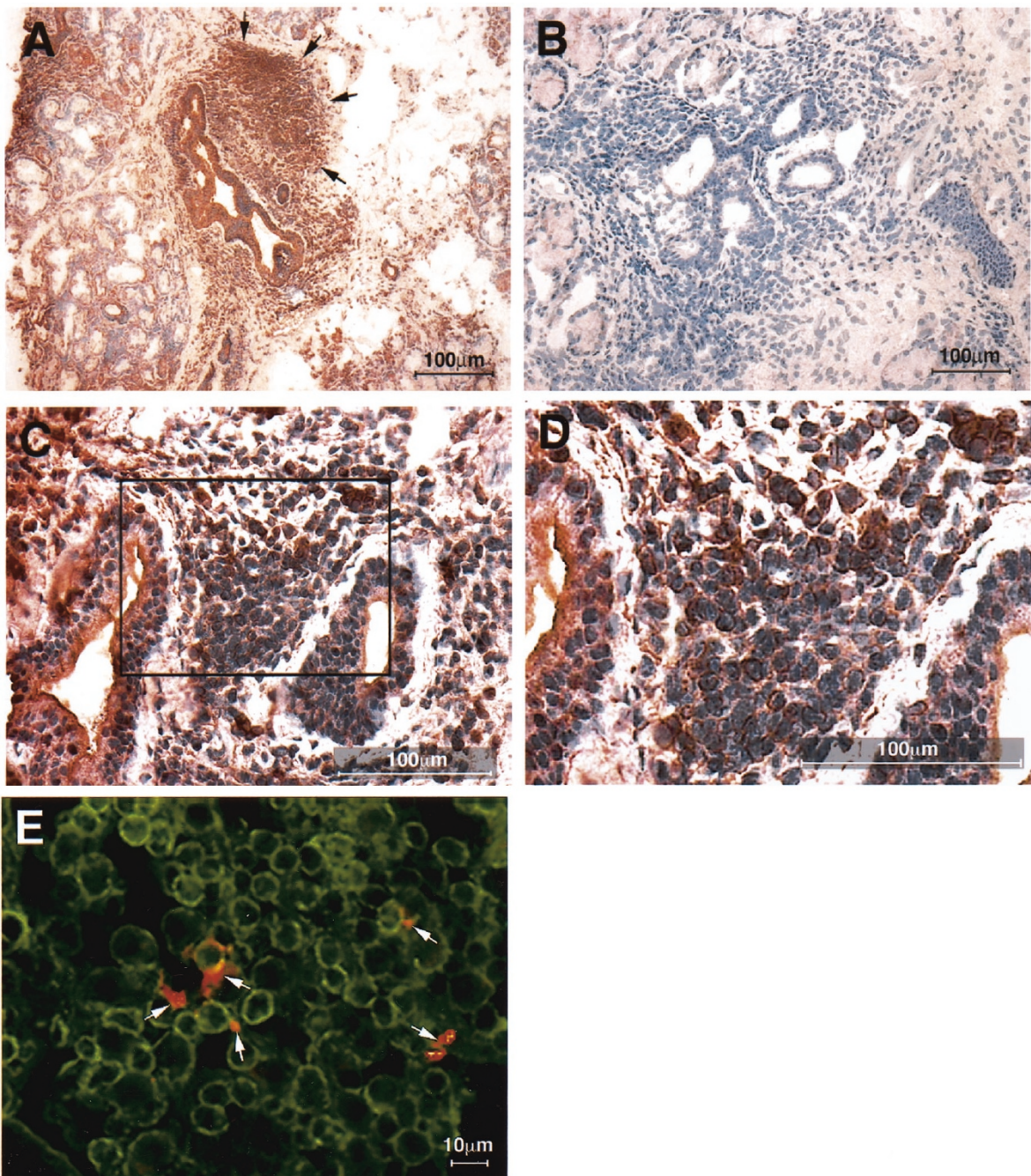


Figure 3.

BAFF expression was detected in situ in salivary glands from patients with SS by application of immunohistochemistry. A, BAFF staining appeared predominantly within salivary gland infiltrates (*arrowheads*) ($\times 20$). B, Negative control of BAFF (concentration-matched rat IgM) represents no specific stainings ($\times 20$). C, BAFF staining appears predominantly on SS infiltrates (*rectangle*) ($\times 40$). D, A higher magnification of the area within the *rectangle* in C ($\times 100$). Membrane-bound staining of BAFF, predominantly on tissue-infiltrating cells and also positive stainings within ductal epithelium. E, Fluorescent immunohistochemical staining for BAFF (green, FITC dye) and TUNEL staining (red, rhodamine) for the detection of in situ apoptosis (*arrowheads*). Images were captured from the same area for green and red emission, then merged with Photoshop. Membrane-bound BAFF⁺ signal appeared frequently on tissue infiltrates. TUNEL-positive apoptotic bodies of BAFF⁺ cells (*arrowheads*, red emission)

numbers were nonsignificantly elevated in SS-SG, underlying an increased BAFF expression in this tissue compared with controls.

Because BAFF is expressed by monocytes and macrophages (CD68) (Moore et al, 1999; Schneider et al, 1999; Shu et al, 1999), we performed a fluorescent double staining for BAFF and CD68 and observed that

these two markers are coexpressed (the frequency of macrophages within tissue infiltrating cells was 7–9%; not shown). Furthermore, for CD68, we found a weaker staining within the ductal and acinar epithelium, and double staining showed that BAFF and CD68 were coexpressed, underscoring the BAFF expression on tissue macrophages infiltrating the epithelium.

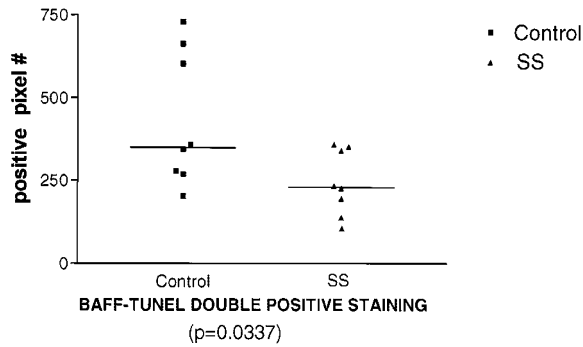


Figure 4.

TUNEL-BAFF double-positive pixel numbers. Each dot represents an individual control/SS patient. Values were calculated as the average positive pixel numbers of three areas.

CD21⁺ B-cell counts were nonsignificantly elevated in primary SS-SG tissue compared with controls. Quantification of the CD21⁺ lymphocytes showed that 17% to 20% of the infiltrating cells were CD21⁺ B cells (not shown), which was in accordance with previous studies (Halse et al, 1999). Our study revealed that CD21 and BAFF colocalized, and we interpret this as the interaction between a BAFF-expressing macrophage and a CD21-expressing B cell as our mAb detects the membrane-bound form of BAFF.

Furthermore, because CD40, Fas, Bcl-2, and Bax can be expressed by salivary gland macrophages, we evaluated the coexpression of BAFF and these pro- and antiapoptotic molecules on the same cells. Apoptosis was assessed by using the TUNEL method among BAFF-expressing cells. Our results showed that Bax and BAFF double-positive pixel numbers were nonsignificantly decreased among patients, suggesting a lower expression of this proapoptotic molecule. This observation corresponds with our further finding that a significantly lower level of apoptosis among the BAFF-expressing cells was detected in salivary glands from patients with SS compared with controls.

Although it has been found that CD40 plays a role in monocyte/macrophage-mediated inflammatory processes by up-regulating cytokine production and promoting rescue from death at sites of inflammation (Grewal and Flavell, 1998), we did not find higher levels of BAFF and antiapoptotic CD40 colocalization in SS-SG compared with controls. These findings suggest that CD40 is not an essential regulator of BAFF⁺ cells.

Interestingly, we observed that in tissue infiltrates BAFF⁺ cells appeared sparsely, although these cells seemed to have the ability to gather in groups or to form a germinal center-like morphology. Germinal center-like structures in SS have previously been described (Amft et al, 2001). Salomonsson et al (2002) identified the lymphoid tissue homing chemokine CXCL13 (BCA-1/BLC) in SS-SG, which suggests that the target organ plays an essential role in the inflammatory process by recruiting B cells and subsets of activated T cells. Furthermore, local production of

autoantibodies against Ro52, Ro60, and La proteins within the salivary gland inflammatory infiltrates was found to be correlated with the presence of high serum levels of the respective autoantibodies (Salomonsson et al, 2002). Specific autoantibody production against Ro52, Ro60, and La might be facilitated by subcellular redistribution and surface exposure of these autoantigens (Ohlsson et al, 2002).

Studies on murine lupus suggest that the interaction between BAFF and its receptors might be important in the pathogenesis of this disease, and studies of BAFF Tg mice have shown that it promotes the production of autoantibodies (Jonsson et al, 2001a). Significant elevations of BAFF partially associated with higher levels of anti-dsDNA Ab of the IgG, IgM, and IgA isotypes have also been described in patients with systemic lupus erythematosus (Zhang et al, 2001). In another study, levels of circulating BAFF in sera from patients with SS were significantly elevated compared with healthy control individuals (Groom et al, 2002). This study also revealed an interesting parallel between an SS-like pathology that emerges in BAFF Tg mice and an imbalance in BAFF production. This implies that an imbalance in BAFF production could be a major factor contributing to the development of SS. BAFF specifically binds to B lymphocytes, costimulates their proliferation and differentiation, and promotes the survival of splenic B cells *in vitro* (Batten et al, 2000).

In SS, B cells are recruited to the salivary glands from peripheral blood by specific lymphoid tissue homing cytokines (Salomonsson et al, 2002). In salivary glands, resident BAFF-expressing cells have the ability to provide signals of proliferation and differentiation to BAFF receptor-bearing B cells and perpetuate the humoral autoimmune process. We found that the frequency of programmed cell death in primary SS BAFF⁺ cells is significantly lower than in healthy individuals, suggesting a prolonged BAFF expression in primary SS-SG. Further investigations showed that this antiapoptotic process is not related to decreased Fas expression and does not show correlation with Bcl-2, Bax, or CD40 coexpression. Because we performed static, slide-based experiments and not functional tests in this study, we could only get information from single time point of this apoptotic process. Therefore, the significantly lower level of apoptotic cells among the BAFF-expressing cells detected in SS-SG could alternatively be explained by the increased clearance of these cells.

It is noteworthy that patients with SS have an increased risk of developing monoclonal B cell non-Hodgkin's lymphoma (NHL) marginal zone-like lymphoma, which frequently occur in the salivary glands (Martin et al, 2000; Thieblemont et al, 1995). The SS-associated lymphoproliferation ranges from essentially benign lesions through mucosa-associated lymphatic tissue-type lesions to frankly dysplastic lymphomas (Burke, 1999). A notable histologic feature in SS, which is also seen in various other autoimmune pathologies (particularly autoimmune thyroiditis), is the lymphoid follicle-like structures with germinal cen-

ters that resemble the architecture of peripheral lymph nodes (Mackay and Rose, 2001). B cells from NHL of multiple histologic subtypes constitutively express the receptor for BAFF to differing levels according to the lymphoma subtype. In addition, patients with follicular NHL have increased levels of soluble BAFF in their serum (Briones et al, 2002). Because the risk of developing B-cell mucosa-associated lymphatic tissue lymphoma in SS is increased compared with the general population and is characterized by B-cell proliferation, this raises the question of whether BAFF may have a role in the lymphoma genesis, in which salivary gland germinal center formation can be a pre-step in the development of B-cell NHL.

We conclude that in SS-SG, an attenuated apoptotic process of BAFF⁺ cells take place, which may cause a longer-existing BAFF-mediated signal for B cells. Prolonged survival of autoreactive B cells might lead to an excessive autoantibody production, maintaining the humoral autoimmune machinery. Furthermore, the increased and continuous BAFF signaling might lead to ectopic germinal center formations or, later, the development of B-cell lymphoma in these patients.

Materials and Methods

Patients and Controls

Minor submucosal labial salivary gland biopsy specimens from eight patients with primary SS and eight healthy controls were obtained from the laboratory's salivary gland biopsy bank and stored at -70°C . Patients fulfilled the revised European classification criteria for primary SS (Vitali et al, 2002) with a focus score higher than 1, whereas controls did not show the characteristic histologic features of primary SS.

Immunofluorescent Staining

Immunohistochemistry was performed on frozen $5\text{-}\mu\text{m}$ tissue sections. After stepwise fixation in 50% acetone at 4°C for 30 seconds and 100% acetone at 4°C for 5 minutes, nonspecific binding was blocked by incubation with 5% goat sera in Tris-buffered saline (TBS) (CD40, CD95, CD21, Bcl-2, CD68, BAFF staining/Alexa Fluor 594), 5% swine sera in TBS (Bax staining), or 5% rabbit sera in TBS (BAFF staining/FITC) for 30 minutes. Blocking solution for double staining contained both respective blocking sera. The sections were then incubated for 60 minutes with monoclonal rat anti-human BAFF (IgM, clone Buffy-2; Alexis Biochemicals, Kelab Göteborg, Sweden; diluted 1/50) in 10% TBS/FCS together with any of the following primary Ab reagents diluted in 10% TBS/FCS: mouse anti-human CD40 (IgG1, clone EA-5; Calbiochem-Novabiochem Corporation, San Diego, California; diluted 1/50); mouse anti-human CD95/FAS (IgM, clone CH-11; Immunotech, Marseille, France; diluted 1/25); mouse anti-human CD21 (IgG1, clone 1 F8; DAKO A/S, Glostrup, Denmark; diluted 1/20); mouse anti-human Bcl-2 (IgG1, clone 124; DAKO A/S; diluted 1/40); mouse anti-human CD68/

macrophage (IgG1, clone KP1; DAKO A/S; diluted 1/50); or polyclonal rabbit IgG anti-human Bax (Santa Cruz Biotechnology, Santa Cruz, California; diluted 1/200).

Fluorochrome-conjugated secondary reagents were then applied for 60 minutes: Alexa Fluor 594 goat anti-rat (IgG; Molecular Probes Europe BV, Leiden, The Netherlands; diluted 1/400 in 5% goat sera/TBS) for BAFF staining, together with FITC-conjugated goat anti-mouse (IgG; Sigma Chemical Company, St. Louis, Missouri; diluted 1/50 in 5% goat sera/TBS) for CD40, CD95, CD21, Bcl-2, and CD68 staining or with FITC-conjugated swine anti-rabbit (IgG; DAKO A/S; diluted 1/30 in 5% swine sera/TBS) for Bax staining. Sections were counterstained with Mayer's hematoxylin for 10 seconds and mounted with aqueous Slow Fade antifade medium (Molecular Probes Europe BV).

All procedures were performed at room temperature. Except for the protein-blocking step, the sections were washed with TBS for 10 minutes after each incubation. Incubations with isotype and concentration-matched immunoglobulins diluted in the respective blocking solutions (omitting the primary Abs) were performed as negative controls: mouse IgG for CD40, CD21, Bcl-2, and CD68 staining; rat IgM for BAFF staining; rabbit IgG for Bax staining; and mouse IgM for CD95 staining.

Immunohistochemical Staining

BAFF, CD40, CD21, CD95, Bcl-2, and Bax expression was evaluated on frozen $5\text{-}\mu\text{m}$ sections with the avidin biotin complex method. Briefly, after stepwise fixation in 50% acetone at 4°C for 30 seconds and 100% acetone at 4°C for 5 minutes, endogenous peroxidase activity was quenched with 0.3% H_2O_2 in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 minutes. To avoid background staining from endogenous biotin, sections were treated with avidin D and biotin blocking solution (Vector Laboratories, Burlingame, California) for 15 minutes each. Nonspecific binding was blocked by incubation for 30 minutes with either 5% normal rabbit serum/5% BSA in TBS (CD40, CD21, Bcl-2, CD68, and CD95 staining), 4% normal mouse serum/4% BSA in TBS (BAFF staining), or 4% normal swine serum/5% BSA in TBS (Bax staining).

The sections were then incubated for 60 minutes with any of the following primary Ab reagents diluted in TBS: anti-human BAFF, anti-human CD40, anti-human CD95, anti-human CD21, anti-human Bcl-2, anti-human CD68, or anti-human Bax. Dilutions, clones, and companies were identical with those described under "Immunofluorescent Staining."

Biotinylated secondary reagents were then applied for 30 minutes: rabbit anti-mouse IgG (DAKO A/S; diluted 1/200) for CD21, CD40, CD68, Bcl-2, and CD95 staining; swine anti-rabbit IgG (DAKO A/S; diluted 1/300) for Bax staining; and mouse anti-rat IgM (BD PharMingen, Laborel, Oslo, Norway; diluted 1/75) for BAFF staining. Abs were all diluted in the respective blocking solutions described above.

Binding of secondary reagents was detected by incubation with $5\ \mu\text{l}$ of avidin DH + $5\ \mu\text{l}$ of biotin

peroxidase in 625 μ l of TBS (Avidin-Biotin Solution Kit; DAKO) for 60 minutes. Staining was finally developed with a substrate consisting of 10 mg of 3-amino-9-ethylcarbazol in 6 ml of dimethyl sulfoxide in 50 ml of 0.02 M NaAc (pH 5.5) + 4 μ l of 30% H₂O₂ buffer for 15 minutes. Sections were counterstained with Mayer's hematoxylin for 10 seconds and mounted with aqueous mounting medium. All procedures were performed at room temperature. Except for the protein-blocking step, the sections were washed with TBS twice for 5 minutes after each incubation. Incubations with isotype and concentration-matched mouse IgG for CD21, CD40, Bcl-2, and CD68, mouse IgM for CD95, rabbit IgG for Bax, and rat IgM for BAFF diluted in the respective blocking solutions were performed as negative controls.

Simultaneous Immunofluorescent Staining for BAFF and Detection of Apoptotic Cells

Immunofluorescent staining and TUNEL labeling was performed on frozen 5- μ m tissue sections. Sections were fixed in fresh 4% paraformaldehyde at room temperature for 15 minutes. After fixation we performed immunofluorescent staining for BAFF, as described in detail above (omitting fixation); as a secondary Ab, we used FITC-conjugated rabbit anti-rat (IgG; Sigma; diluted 1/200). After stepwise staining, we performed a fluorescent TUNEL labeling of apoptotic cells as described previously (Ohlsson et al, 2002). Briefly, sections were washed in PBS and equilibrated at 37° C for 2 \times 5 minutes in terminal-deoxynucleotidyl transferase (TdT) buffer (0.5 M cacodylate, pH 6.8, 1 mM CoCl₂, 0.5 mM dithiothreitol, 0.05% BSA, and 0.15 M NaCl). Incubation with TdT buffer containing 0.1 U/L TdT (Boehringer Mannheim, Mannheim, Germany) and 8 nmol/ml digoxigenin-conjugated dUTP (Boehringer Mannheim) was then performed in a humidified chamber for 1 hour at 37° C. Negative controls were incubated with reaction buffer alone, omitting TdT and DIG-dUTP from the solution. The reaction was stopped by washing in PBS. TUNEL-positive cells were labeled with sheep anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics, Oslo, Norway; diluted 1/200 in 2% BSA/PBS) for 30 minutes at room temperature. Sections were counterstained with Mayer's hematoxylin for 10 seconds, and the cells were finally mounted with aqueous Slow Fade antifade medium.

Specificity of Anti-BAFF mAb for Salivary Gland-Expressed BAFF

Immunohistochemistry was performed on frozen 5- μ m tissue sections. After stepwise fixation in 50% acetone at 4° C for 30 seconds and 100% acetone at 4° C for 5 minutes, nonspecific binding was blocked by incubation with 5% goat sera in TBS for 30 minutes. The sections were then incubated for 60 minutes with monoclonal rat anti-human BAFF alone (IgM, clone Buffy-2; Alexis Biochemicals; diluted 1/50) in 10% TBS/FCS or together with different concentra-

tions of recombinant human soluble BAFF (purified rhsBAFF; amino acid 83–285; Alexis Biochemicals; diluted 1/100 = 0.001 mg/ml, 1/50 = 0.002 mg/ml, 1/25 = 0.004 mg/ml, 1/5 = 0.02 mg/ml) to evaluate the staining-inhibitory capability of the recombinant protein in a dose-dependent manner.

Fluorochrome-conjugated secondary reagents were then applied for 60 minutes (Alexa Fluor 594 goat anti-rat; IgG; Molecular Probes Europe BV; diluted 1/400). Sections were counterstained with Mayer's hematoxylin for 10 seconds and mounted with aqueous Slow Fade antifade medium (Molecular Probes Europe BV).

All procedures were performed at room temperature. Except for the protein-blocking step, the sections were washed with TBS for 10 minutes after each incubation. Incubation with isotype- and concentration-matched rat IgM diluted in the respective blocking solution (omitting the primary Ab) was also performed as a negative control. Evaluation of the slides was performed using fluorescent microscopy according to the method described below.

Quantitation of Fluorescent Signals

Fluorescent images from the green and red emission spectra and normal light microscopy images were captured from the same area with a fluorescent Leica DC 100 microscope (Leica Microsystems, Wetzlar, Germany) with a \times 20 objective. Three different regions were randomly selected on each slide for analysis.

To quantify the fluorescence pixels and pixel luminosity values, we used Photoshop 6.0.1 software. Two images from the same position on the slide, one with red fluorescence and one with green fluorescence, were loaded into Photoshop and converted to grayscale 8-bit images with pixel luminosities ranging from 0 to 255.

Positive fluorescence was defined as pixel values with a minimum luminosity. The threshold values were set by the Photoshop threshold function at the end slope of the normal distribution of the background pixel luminosities. Both images were then merged into an RGB picture, in which the red channel was represented by the red fluorescence grayscale image and the green channel by the green fluorescence grayscale image. An empty grayscale 8-bit image (luminosity 0) was created and selected as the blue channel. In this RGB image, red color would be present where there was only red fluorescence and green color where there was only green fluorescence; yellow color would indicate double-positive pixels. This enabled selection of pixels in one of the images and switching to one of the other images within the same selection. From this RGB image, pixels with a certain RGB value for the specific color were selected by using the Select Range function in Photoshop. Thereafter it was possible to switch to the original fluorescence image and get information about the number of selected pixels and the mean luminosity and standard deviation of the pixels by using the histogram function

in Photoshop. This was done for the colors red, green, and yellow. When measuring the mean luminosity for double-positive cells, we combined the original red fluorescence and green fluorescence pictures into a new image before using the histogram function. Finally, to identify the localization of positive signals, red and green images were merged with identically located light microscopy pictures (Fig. 2).

The Interpretation of Positive Pixel Numbers and Luminosity Intensity Values

The different fluorescent pixel numbers correlate with the numbers of positive cells, whereas luminosity intensity data refer to the marker density of the cells. When fluorescent images were overlaid on the corresponding hematoxylin-stained light microscopy pictures, a homogenous pixel-distribution within stained cells was found. The intensity of the light emission from one pixel is proportional with the amount of the fluorescent dye molecules that can bind to this region represented by the pixel. Because the amount of bound fluorochrome-conjugated secondary Ab is proportional with the target cell molecule numbers, the monochromatic light emission from one pixel is proportional with the antigens density of the target.

Statistical Analysis

Statistical differences in measured values representing either staining intensity or staining area were analyzed using Student's *t* test. A *p* value < 0.05 was considered significant.

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