Sjogren's syndrome
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Chapter 5b
Clinical and histologic evidence of salivary gland restoration supports the efficacy of rituximab treatment in Sjögren’s syndrome
Abstract

**Objective** To assess the effect of rituximab (anti-CD20 antibody) therapy on the (immuno)histopathology of parotid tissue in patients with primary Sjögren’s syndrome (pSS) and the correlation of histologic findings with the flow rate and composition of parotid saliva.

**Methods** In a phase II study, an incisional parotid biopsy specimen was obtained from 5 patients with pSS before and 12 weeks after rituximab treatment (4 infusions of 375 mg/m2). The relative amount of parotid parenchyma, lymphocytic infiltrate and fat, and the presence/quantity of germinal centers and lymphoepithelial duct lesions were evaluated. Immunohistochemical characterization was performed to analyze B:T cell ratio of the lymphocytic infiltrate (CD20, CD79a, CD3) and cellular proliferation in the acinar parenchyma (by double immunohistologic labeling for cytokeratin 14 and Ki-67). Histologic data were correlated to parotid flow rate and saliva composition.

**Results** Four patients showed an increased salivary flow rate and normalization of the initially increased salivary sodium concentration. Following rituximab treatment, the lymphocytic infiltrate was reduced, with a decreased B:T cell ratio and (partial) disappearance of germinal centers. The amount and extent of lymphoepithelial duct lesions decreased in 3 patients and was completely absent in 2 patients. The initially increased proliferation of acinar parenchyma in response to the inflammation was reduced in all patients.

**Conclusion** Sequential parotid biopsy specimens obtained from patients with pSS before and after rituximab treatment demonstrated histopathologic evidence of reduced glandular inflammation and redifferentiation of lymphoepithelial duct lesions to regular striated ducts as a putative morphologic correlate of increased parotid flow and normalization of salivary sodium content. These histopathologic findings in few patients underline the efficacy of B cell depletion and indicate the potential for glandular restoration in SS.
Introduction

Currently, there is no evidence-based intervention treatment for Sjögren’s syndrome (SS), but biologic agents are promising.(1) Rituximab, a chimeric murine/human anti-CD20 monoclonal antibody that binds to the B-cell surface antigen CD20, is a well-established therapeutic agent in the treatment of B-cell non-Hodgkin lymphomas, and is a new promising therapeutic modality in different autoimmune disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematosus.(2)

The salivary glands of patients with SS are histologically characterized by lymphocytic infiltration with progressive parenchymal atrophy and formation of the characteristic lymphoepithelial lesions in striated ducts, formerly called “epimyoepithelial lesions”.(3) Our group has previously shown that lymphoepithelial lesions develop from basal cells of striated ducts, representing an aberrant metaplastic differentiation, triggered by the epitheliotropic autoimmune inflammation in SS.(3) In parallel, parenchymal acinar cells in SS demonstrate increased proliferation in an effort to partially compensate for enhanced apoptotic cell loss.(3-5) Our group previously reported clinical data from a phase II trial with rituximab treatment in 8 patients with primary SS, which showed significant improvement of subjective symptoms and increased salivary secretion with partial normalization of increased sodium concentration of saliva in patients with early-onset SS.(6) These findings might indicate partial recovery of salivary gland tissue.(7) In 5 of the 8 patients with pSS involved in the above-mentioned study, sequential parotid gland biopsy specimens were available for histologic analysis; these specimens were obtained before and 12 weeks after rituximab treatment. In the other 3 patients with pSS no second biopsy specimen was obtained, because these patients did not complete rituximab treatment due to the development of serum sickness.(6)

The biopsy material gave us the unique opportunity to correlate clinical findings, including the salivary flow rate and composition of saliva, with the findings of a detailed immunohistopathologic analysis of the parotid gland biopsy specimens obtained before and after rituximab treatment in order to histologically verify the effects of therapeutic B cell depletion in patients with SS.
Patients and methods

Study design
Five female patients (mean age 53 years, range 43-65 years), all of whom fulfilled the American-European consensus criteria for pSS, were treated with 4 infusions rituximab (Roche, Woerden, the Netherlands), given at a dosage of 375 mg/m2/week. No other immunosuppressive therapy was used. An incisional biopsy specimen of the parotid gland was obtained from the same gland before and 12 weeks after therapy. These patients were part of an earlier reported phase II trial.

Parotid gland function and salivary composition
Unstimulated and stimulated parotid saliva was collected in a standardized way at baseline and 12 weeks after treatment as described previously. Flow rates were calculated and sialochemical analysis was performed, focusing on the concentration of sodium in parotid saliva, particularly because increased sodium in parotid saliva is indicative of SS and reflects damage to the ductal system. High levels of sodium in the saliva of patients with SS are associated with higher levels of disease activity and a more progressive course of the disease.

Histopathologic analysis
Biopsy specimens were fixed in 4% neutral buffered formalin, embedded in paraffin, cut at a thickness of 3 μm, and stained with hematoxylin and eosin. The relative amount of glandular parenchyma, lymphocytic infiltrate, and fat was assessed semiquantitatively in steps of 10%, each in relation to the total amount of biopsied parotid tissue. The presence of secondary germinal centers was assessed as follows: 0 = no germinal centers, I = few (mostly small) germinal centers, and II = many (often large) germinal centers. The characteristic ductal alterations of SS (lymphoepithelial lesions) were evaluated by the following grading system: 0 = none, I = few and partially developed lymphoepithelial lesions (not circumferential, <50% of all striated ducts) and II = fully developed lymphoepithelial lesions (fully circumferential, >50% of all striated ducts). Biopsy specimens were independently scored as based on these criteria by 2 investigators (J.P. and S.I.) in a blinded manner. In case of discrepancy a definite score was determined by consensus.

Immunohistochemical analysis
In representative areas of lymphocytic infiltrates the numbers of B cells (staining for CD20) and T cells (CD3) were quantified in 1000 lymphocytes each, and consecutively calculated as B/T cell ratio. To evaluate a possible additional down-regulation of CD20 antigen presentation on persisting B cells due to anti-CD20 therapy, quantification of B cells was separately performed with antibodies to CD20 and CD79a. Due to technical limitations, it is not possible to quantify the absolute amount of B and T cells.

As described previously, a double immunohistochemical labeling technique for cytokeratin 14 (CK14) (labeling basal cells of striated ducts and myoepithelial cells) and Ki67 (labeling cellular proliferation) greatly enhances the exact identification and quantification of cellular proliferation of the various epithelial cells of the gland. In order to evaluate the regenerative potential of the glandular parenchyma, cellular proliferation in the CK14-negative acinar cells was calculated in representative areas of lymphocytic infiltration (nuclear positivity for Ki67 as a percentage of 400 acinar cells). For staining of
Figure 1
Comparison of parotid biopsy specimens obtained from patient 3 before therapy (left, figures 1A1-4) and 12 weeks after therapy (right, figure 1B1-4). Magnification: 120x figures 1A1,1B1; 100x figures 1A2,1B2; 60x figures 1A3,1B3; 200x figures 1A4,1B4. Figure 1A1: Before treatment, double staining illustrates intense inflammation (arrows) with highly proliferating, large germinal centres (GS; Ki67 with nuclear staining), fully developed lymphoepithelial lesions (LEL; CK14 staining) and reduced glandular parenchyma (PAR). After therapy (figure 1B1), inflammation is reduced (arrows) with absence of germinal centres and presence of regular striated ducts (SD) devoid of lymphoepithelial lesions. Before therapy there was a dominance of B lymphocytes with germinal centres (GS; figure 1A2: CD20) in comparison to T lymphocytes (inset: figure 1A3: CD3). After therapy the overall reduced lymphoid infiltrate with slight dominance of T lymphocytes (inset: figure 1B3: CD3) in comparison to B lymphocytes (figure 1B2: CD20). In higher magnification (figure 1A4) fully developed lymphoepithelial lesions, many intraepithelial lymphocytes and increased basal cell proliferation (arrows), contrasting after therapy to regular striated duct with CK14-positive basal cells (arrows in figure 1B4) with regular differentiation into luminal ductal cells, devoid of intraepithelial lymphocytes (arrowheads).
**Chapter 5b**

**Table 1** Clinical and (immuno-)histological data before and after rituximab therapy.

<table>
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<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
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<td>Parotid flow</td>
<td>0.14</td>
<td>0.16</td>
<td>0.15</td>
<td>0.21</td>
<td>0.18</td>
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<td>Na+ in parotid saliva</td>
<td>39 (5)</td>
<td>27 (2)</td>
<td>12 (5)</td>
<td>7 (7)</td>
<td>19 (6)</td>
<td>8 (7)</td>
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<td></td>
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<td>Parenchyma (%)*</td>
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<td>70-80</td>
<td>40-50</td>
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<td>Lymphocytic infiltrate (%)*</td>
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<td>10-20</td>
<td>10-20</td>
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<td>Fat (%)*</td>
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<td>10-20</td>
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<td>Germinal centres</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>No</td>
<td>II</td>
<td>No</td>
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<tr>
<td>Lymphoepithelial duct lesions (LEL)</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>No</td>
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<td>Proliferation of acinar parenchyma in % (Ki67)</td>
<td>3.8</td>
<td>3.2</td>
<td>3.4</td>
<td>2.3</td>
<td>3.5</td>
<td>2.5</td>
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<td>B :T cell ratio (CD20/CD3)</td>
<td>76/24</td>
<td>67/33</td>
<td>59/41</td>
<td>28/72</td>
<td>58/42</td>
<td>35/65</td>
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Parotid flow: stimulated parotid secretion (ml/min); Na+: concentration of sodium in parotid saliva (mmol/l), value in brackets: sodium concentration to be expected in healthy subjects with the given parotid flow; N.A.: not available; * Percentages in steps of 10% represent assessment of the area of the biopsy specimen. Germinal centres, I = few germinal centres, II = many germinal centres; Lymphoepithelial duct lesions, I = partially developed lymphoepithelial lesions (not circumferential, <50% of all ducts), II = fully developed lymphoepithelial lesions (fully circumferential, >50% of all ducts); B:T cell ratio: ratio of B and T lymphocytes in the infiltrate; ↑: increase, ↓: decrease.
CK14 an avidin-biotin-peroxidase method was applied (ABC kit; Vector, Burlingame, CA), for staining of Ki67, the alkaline phosphatase-anti-alkaline phosphatase method was used (APAAP-ChemMate; Dako Cambridge, UK).

Results

The clinical and (immuno)histologic data for biopsy specimens obtained before and after rituximab treatment are summarized in table 1. All patients showed a clinical response as reflected, among other factors, by significant improvement of subjective symptoms. Four of 5 patients showed a minor-to-moderate increase in the parotid flow rate (mean increase 24%). The baseline sodium concentration in parotid saliva was increased in the saliva samples from these 4 patients (patient 5 had no salivary parotid flow at baseline) (table 1). The sodium concentration decreased after treatment in all 4 of the above-mentioned patients, and values returned to near normal in 2 of these 4 patients.

The histologic data showed a tendency towards reduced lymphocytic infiltration after therapy with a decrease of the B:T cell ratio, indicating a major decrease especially in the number of B lymphocytes, in combination with a reduction of germinal centers (which were completely absent in 4 patients). The number of B lymphocytes based on staining for CD20 and CD79a did not differ. The amount of acinar parenchyma did not change or was slightly decreased, and the amount of fat did not change or was increased. Parallel to the reduction in the number of intraepithelial lymphocytes, the amount and extent of lymphoepithelial lesions decreased in 3 of 5 patients, and these lesions were completely absent in 2 of 5 cases. Cellular proliferation of acinar parenchyma before therapy was higher (average 3.4% figure 2A) than that of normal acinar parenchyma of patients without SS (2.0%,5), and was found to be reduced in all patients after therapy (on average 2.5%). The most significant improvement of clinical and histological findings was observed in patient 3. (as shown in figures 1 and 2A). Statistical correlation of the different parameters could not be determined due to the small sample size.

Discussion

Rituximab is a promising treatment option for patients with pSS and systemic complications and/or active and progressive disease, but more data from randomized controlled trials are warranted before more accurate conclusions on the role of rituximab can be made.(10) This study is the first to present histologic data demonstrating evidence of a reduction in glandular inflammation combined with signs of partial glandular restoration, parallel to increased parotid saliva flow and normalization of initially increased levels of salivary sodium. As expected, the reduction of inflammation was mainly attributable to a depletion of B lymphocytes, as has been previously described following rituximab therapy in RA.(11) Although quantification of the absolute amount of B and T cells was not possible for technical reasons, the overall decrease in the amount of infiltrate, combined with a decreased B:T cell ratio, suggests a relevant decrease in the amount of B cells. The preponderant absence of germinal centers and the reduction of intraepithelial lymphocytes in the salivary ducts after therapy underline the significant reduction of inflammatory activity. This correlates to complete depletion of B cells in the peripheral blood 12 weeks
after start of treatment (6), comparable to data from a recent study in patients with RA.(12) In addition, also T lymphocytes seemed to decrease slightly after therapy, although this could not be quantified.

Although the number of B cells in parotid gland tissue was decreased, B cells were not completely depleted. The discrepancy with complete B cell depletion observed in peripheral blood might be explained by the expression of different protective factors in this tissue, such as BLYS (B lymphocyte stimulator) or BAFF (B cell activating factor). The same phenomenon has been observed in patients with RA treated with rituximab.(12) In contrast, another study of SS patients showed a complete depletion of B cells in labial salivary glands 4 months after rituximab treatment.(13) Possible explanations for this difference might be the increased inflammatory activity in parotid salivary glands (reflected by germinal centers) or a difference in the expression of BAFF or BLYS. Further studies are necessary to investigate the different B cell subsets before and after treatment and the expression of BAFF or BLYS.

The widespread presence of fully developed lymphoepithelial lesions before therapy and the reduction or complete disappearance of lymphoepithelial lesions after therapy offer histopathologic evidence that fully developed lymphoepithelial lesions can completely redifferentiate into regular striated ducts (see figure 2B). As shown previously by our group lymphoepithelial lesions in SS develop from enhanced proliferation of basal cells of striated ducts with an aberrant metaplastic lymphoepithelial differentiation, triggered by the epitheliotropic autoimmune inflammation.(3,14) Supposedly, this redifferentiation into regular striated ducts after therapy is recruited from surviving and proliferating basal cells in lymphoepithelial lesions with physiological differentiation into regular ductal cells (figure 1B4).

**Figure 2**
A. Minor increase of acinar cell proliferation (arrows) as demonstrated by double staining with CK14-Ki67, adjacent to lymphocytic infiltration with lymphoepithelial lesions (LELs) and germinal centre (GS; patient 3 prior to therapy; magnification 200x).  
B. Schematic illustration of partially reversible glandular alterations in SS: Black arrows (bottom) indicate transformation of striated duct (left) into incomplete (middle) and fully developed lymphoepithelial lesions (right), in addition to progressive loss of acini and intercalated ducts (black arrows top). Grey arrows (bottom) illustrate evidence of complete redifferentiation of fully developed lymphoepithelial lesions to regular striated ducts after therapy. Effective regeneration of intercalated ducts and acini as an effect of successful Rituximab therapy is hypothetical (dotted grey arrows).
In healthy subjects, most of the high sodium content in primary saliva is actively reabsorbed during passage through striated ducts. The increased sodium content in saliva of patients with pSS has been attributed to severely impaired reabsorption in the structurally altered lymphoepithelial lesions. Reduction or normalization, respectively, of the salivary sodium concentration after B cell depletion obviously is attributable to partial or complete redifferentiation of lymphoepithelial lesions to regular striated ducts, with reconstituted physiological function, including regular reabsorption of sodium.

Increased proliferation of acinar parenchyma in pSS in comparison with regular glands has been interpreted as a regenerative effort to compensate for increased apoptotic cell loss in the inflamed parenchyma. Accordingly, the observed minor decrease of proliferation in acinar parenchyma after rituximab treatment of pSS presumably is attributable to a decrease of the inflammatory stimulus. There is no good explanation for the almost absent parotid salivary flow in patient 5, despite the amount of salivary parenchyma (table 1). It has been shown that many patients with SS have, within their salivary glands, large amounts of acinar tissue that is unable to function in vivo, possibly due to antimuscarinic antibodies.

In summary, these findings are the first to provide histopathologic evidence that rituximab treatment in SS can induce reduction of glandular inflammation and structural redifferentiation of lymphoepithelial duct lesions, correlating to a gain in function of the glands, especially with respect to improved function of the structurally redifferentiated striated ducts. The decrease in lymphocytic infiltration, the number of germinal centers, intraepithelial lymphocytes, and acinar proliferation, combined with redifferentiation of lymphoepithelial lesions in 3 patients, suggests efficacy of B cell depletion in salivary glands. A larger placebo-controlled randomized clinical trial investigating the immunohistologic correlation of sequential biopsy specimens obtained before and after therapy has been started by our group in order to prove the findings suggested in this uncontrolled study.
Reference List
