# Immunohistologic markers of immune activation and changes of glycosylation of serum proteins in primary Sjögren's syndrome

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## Abstract

### Objective

*To assess the possible correlations between the immune activation of certain surface antigens at the lip salivary gland (LSG) level, and changes in glycosylation of serum proteins in primary Sjögren's syndrome (SS).*

### Methods

*LSG biopsy samples were obtained from 22 SS patients (mean age 56.3 years; mean disease duration 70.8 months) and prepared for immunohistochemical analysis using murine monoclonal antibodies for interleukin-2 receptor (IL-2R) (CD25) and for the class II major histocompatibility antigen HLA-DR. The glycosylation of serum proteins was evaluated in all patients by an enzyme-linked lectin assay (ELLA) using concanavalin A (Con A).*

### Results

*In LSG specimens the presence of IL-2R was observed at the infiltrating level, mainly periductally, in 13 (59%) cases and on the epithelial cells of 14 (64%) patients. In 13 out of 22 SS patients (59%) a marked positivity both of the infiltrates and of the epithelium was found for anti-HLA-DR monoclonal antibody. The degree of expression of different antigens on LSG samples was correlated with their histologic class according to Tarpley evaluation. The positivity for IL-2R and HLA-DR molecules on glandular tissues was correlated. A significant increase in the total Con A reactivity of serum proteins was found in those patients expressing IL-2R and HLA-DR antigens on LSG specimens.*

### Conclusions

*The co-expression of IL-2R and HLA-DR antigens on both the epithelium and infiltrates of LSG is consistent with a participation of these cells in the immune process of SS. Moreover, changes in the glycosylation of serum proteins seem to be related to the presence of these immunoactivation markers of the disease at the LSG level, suggesting that the control of protein glycosylation could be mediated by the same mechanisms involved in the tissue damage of SS.*

**Key words**

Sjögren's syndrome, lip salivary glands, protein glycosylation.

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## Introduction

Immunopathological studies have shown that almost all the infiltrating elements at lip salivary gland (LSG) level of Sjögren's syndrome (SS) patients are activated and most of them are T cells (1). The activation status of these cells can be evaluated by searching for membrane expression of the surface antigen of interleukin-2-receptor (IL-2R) or for HLA class II molecules, considered to be aspecific markers of immune activation and indices of disease activity in the autoimmune sialoadenitis of SS (2-4). SS ductal and acinar epithelial cells of the LSG inappropriately express HLA-DR antigens (2), whereas the monoclonal antibody anti-CD25, which detects the CD25 molecule (IL-2R), may be expressed not only by the infiltrating lymphocytes, but also by the epithelial cells of LSG in SS (4, 5). These findings suggest that these molecules are not only indicators of lymphocytic activation but also play a role as growth and cell proliferation markers in SS (1, 4).

It has been suggested that a possible cause of autoimmune diseases is the formation of new epitopes as a result of protein denaturation (6) and it is well known that the concentrations of certain proteins increase with inflammation (7). Oligosaccharide structures play a role in the antigenicity of a number of clinically important antigens, and they also have a central function in adhesion and homing events during inflammatory processes (8). Additionally, alterations in glycosylation are known to occur in a wide number of autoimmune diseases (8, 9). In fact, the majority of molecules involved in the immune system are glycosylated (for example, the HLA class II molecules) and glycosylation changes can have profound effects on glycoprotein function (9).

Most studies have focused on the alterations in IgG glycosylation that have been described in many different rheumatological diseases, such as rheumatoid arthritis (RA), juvenile chronic arthritis (JCA), systemic lupus erythematosus (SLE) and SS (9, 10). However, changes in glycosylation have also been reported by us and other authors for other serum glycoproteins, such as 1-acid glycoprotein, 1-antitrypsin, 2-macroglobulin, in many inflammatory conditions (11-15). We hypothesized that these changes could lead to an alteration of the immunoreactivity of these glycoproteins in selected diseases, and postulated that their involvement in specific self-antigen modifications may lie at the basis of the autoimmune response (13).

The lectin concanavalin A (Con A), which is able to detect changes in N-glycan branching, has been used to evaluate alterations in serum glycoproteins (16). Changes in Con A reactivity in both acute phase and non-acute phase proteins are known to occur in various diseases including SLE, systemic sclerosis (SSc), RA and SS (11, 12, 17). Thus, abnormal glycosylation processes could be associated with autoimmunity and the study of changes in glycosylation patterns may be a valuable diagnostic approach to facilitate our understanding of inflammatory and autoimmune diseases.

We recently described a good correlation between changes in the glycosylation of serum proteins and elevated serum levels of sIL-2R in SS patients, indicating that these changes occur more frequently in more severe disease states (18). In the same year a decrease in the production rate and a change in the glycosylation pattern of the tear glycoproteins was found in primary SS (19). Recent data indicate that mannose binding, lectin-related ligands expressed on LSG structures could play distinct roles in the pathogenesis of primary versus secondary SS (20).

T-cells have been shown to produce factors which regulate glycosylation; in fact many cytokines, which are mediators of immunological responses and inflammation, are known to play an important role in controlling the glycosylation of acute phase proteins (21, 22).

To our knowledge, the relationship between changes in glycosylation and the tissue markers of immune activation, such as IL-2R and HLA-DR antigens, which may be expressed on the cell surface in labial biopsy specimens of SS patients, has never been investi-

gated. The aim of this study therefore was to evaluate the possible relationship between the surface antigen expression of immune activation at the LSG level and changes in the glycosylation of serum proteins in primary SS.

## Materials and methods

We studied 22 consecutive patients (M/F 2/20; mean age 56.3 yrs., range 36-77) with primary SS diagnosed according to the modified classification criteria of Vitali *et al.* (23) (mean disease duration 70.8 months, range 12-240). The following parameters were measured in all patients: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), blood cell count, 2-globulins, gammaglobulins, immunoglobulins of the IgG, IgM and IgA isotypes, and C3 and C4 fractions. Antinuclear antibodies (ANA) using indirect immunofluorescence on the Hep-2 cell line (Kallestad, Chaska, USA) were detected. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the serum levels of anti-SSA/Ro and anti-SSB/La antibodies (Inova, San Diego, USA). Rheumatoid factor (RF) was identified by the latex test. We evaluated the total glycosylation of serum proteins by a recently developed enzyme-linked lectin assay (ELLA) using Con A as previously described (24).

23 healthy volunteers matched for sex and age were chosen to serve as normal controls in serum investigations.

After their informed consent was obtained, a LSG biopsy was taken from all patients through normal appearing mucosa in the lower lip. Glandular specimens were prepared as follows: part of the tissues were embedded in Tissue-Tec OCT compound (Miles Laboratories, Naperville, IL, USA), snap-frozen in liquid nitrogen and either cut immediately into 6 thick serial sections or stored at -70° C until used. After cryostatic sectioning, the slices were allowed to dry at room temperature overnight and were then fixed in a chloroform:acetone (1:1, v:v) mixture. The remaining biopsy tissues were fixed in formalin and embedded in paraffin for routine histology. Sections were analyzed to evaluate the infiltrate, using the procedure of Chisolm & Mason (25) and the histological classes proposed by Tarpley *et al*. (26).

Cryostatic sections for each patient were stained using murine monoclonal antibodies for IL-2R (CD25) and for class II major histocompatibility antigen HLA-DR (Becton Dickinson, Sunnyvale, CA, USA). A standard 3-stage immunoperoxidase-labelling technique utilizing avid-biotin-immunoperoxidase complex (ABC) was employed (Lab Vision, Fremont, CA, USA). Briefly, on cryostatic sections endogenous peroxidase activity was inhibited by applying a solution of 3% hydrogen peroxide and 2% normal goat serum to block non-specific uptake. The primary antibody was followed by biotin-labelled anti-serum and then by ABC. The sections were developed in diaminobenzidine-$H_2O_2$ solution, counterstained with Mayer's hematoxilin and finally mounted in glycerol/PBS (27). The same procedures, with substitution of the primary antibody by identical dilutions of non-immune sera, were followed to serve as negative controls.

To assess expression of the markers at the epithelial level and in the infiltrating elements of the salivary glands, the specimens were blindly analyzed by two of the authors and the mean of the two independent observations was recorded. Only cells clearly staining with the antibody were scored as positive. The results were evaluated as follows: 0: the absence of positive cells; 1: < 10% positive cells; 2: 10 - 25% positive cells; 3:25 - 50% positive cells; 4 > 50% positive cells per high power field (HPF) (x400) .

A group of 8 individuals with normal salivary glands and no signs of autoimmune disease served as controls.

*Statistical methods*

Categorical variables were analyzed by Fisher's exact test and continuous variables were analyzed by the Wilcoxon rank test for paired data and by the Mann-Whitney test for unpaired data. The significance of the correlations was determined using the Spearman rank correlation coefficient. P values less than 0.05 were considered significant.

## Results

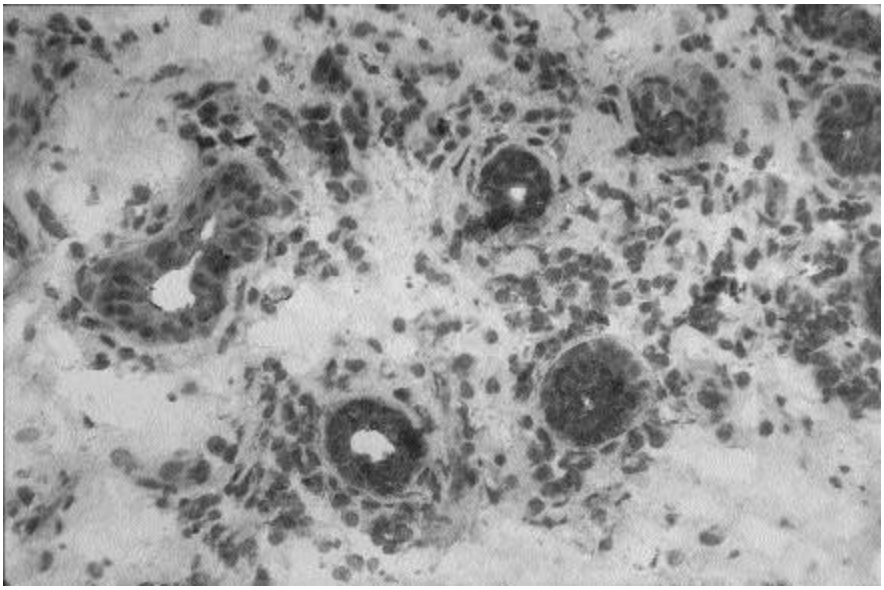
The main clinical and immunological features of the 22 SS patients are shown in Table I. Using the Tarpley scale of 0 to 4, 20 patients (91%) could be classified as class 1 or higher. The presence of IL-2R (CD25+) was observed in the LSG lymphocytic infiltrate of 13 (59%) patients, where the positive infitrating elements were mainly located periductally, while a marked expression of CD25 was found in the LSG epithelium of 14 (64%) subjects (Fig.1 A). A diffuse cytoplasmic positivity of the infiltrating cells and of ductal and acinar cells was seen with anti-HLA-DR monoclonal antibody in 13 out of 22 cases (59%) (Fig.1 B). None of the normal controls stained for these antibodies.

We found a significant correlation between the Tarpley histologic class and the grade of different monoclonal expressions on LSG specimens (CD25 positive infiltrates $p < 0.026$; CD25 positive epithelium $p < 0.059$; HLA-DR positive infiltrates $p < 0.0065$; HLA-DR positive epithelium $p < 0.033$).

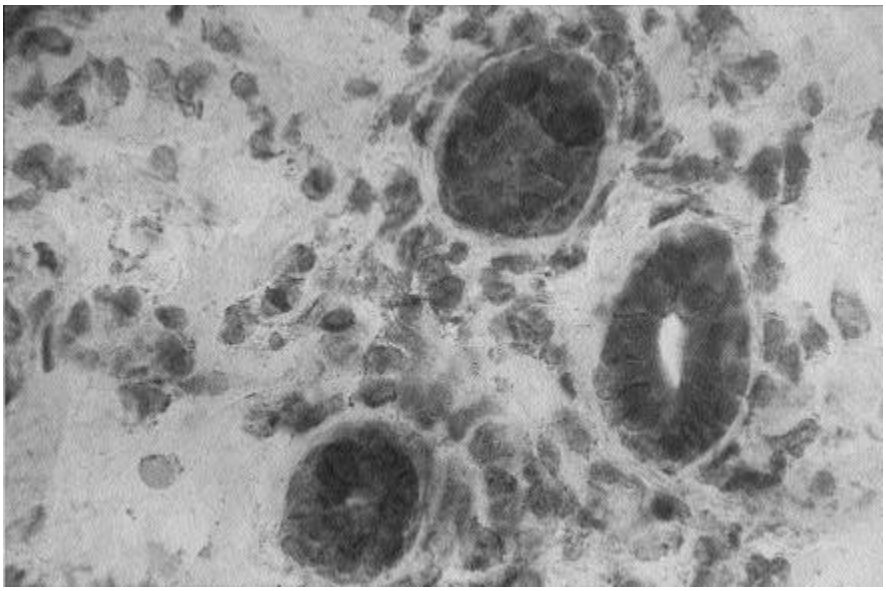
A significant relationship was detected between CD25 expression in the glan-

**Table I.** Main clinical and immunological features of 22 patients with primary Sjögren's sndrome

| | | |
|---|---|---|
| Sex (M/F) | 2/20 | |
| Age (years) | 56.3 (range 36 - 77) | |
| Disease duration (months) | 70.8 (range 12 - 240) | |
| ANA (no., %) | 21 | 95% |
| Anti-SSA/Ro (no., %) | 17 | 77% |
| Anti-SSB/La (no., %) | 11 | 50% |
| Anti-SSA/Ro + anti-SSB/La (no., %) | 11 | 50% |
| IgM-RF (no., %) | 22 | 100% |
| Extraglandular manifestations (no., %) | 16 | 73% |



**(a)**



**(b)**

**Fig. 1.** Immunoperoxidase staining of cryostatic salivary gland sections in Sjögren's syndrome. (**a**) Immunolocalization of anti-IL-2R monoclonal antibody (CD25) at the epithelial level and within the surrounding mononuclear infiltrating cells. Original magnification x 250. (**b**) Immunolocalization of anti-HLA-DR monoclonal antibody showing a diffuse cytoplasmic positivity of ductal,acinar and infiltrating elements. Original magnification x 400.

dular lymphocytic infiltrate and CD25 positivity in the epithelial cells ($p < 0.0002$), and with HLA-DR expression both on lymphocytes ($p < 0.0035$) and in epithelial cells ($p < 0.005$), whereas the presence of this class II molecule in the infiltrating lymphocytes and in the epithelium correlated with each other ($p < 0.0025$).

The values of total Con A reactivity of serum proteins in our SS patients and in normal donors are shown in Figure 2.

SS patients expressing IL-2R on LSG had higher values of Con A affinity (median 1.14; 25th - 75th percentile 0.89 - 1.52 leq) compared to patients not expressing this receptor (median 0.64; 25th - 75th percentile 0.54 - 1.1 leq) ($p < 0.05$) and to normal donors (median 0.83; 25th - 75th percentile 0.69 - 1.02 leq) ($p < 0.02$).

The Con A affinity of serum proteins was also elevated (median 1.14; 25th - 75thpercentile 0.89 - 1.52 leq) in SS patients showing HLA-DR positivity on LSG compared to those specimens without this molecule (median 0.66; 25th - 75th percentile 0.53 - 1.07 leq) ($p < 0.05$) and to normal donors (median 0.83; 25th - 75th percentile 0.69 - 1.02 leq) ($p < 0.02$).

The lack of these markers of immunoactivation on the LSG specimens of our SS patients was not associated with significantly different values for the Con A reactivity of serum proteins with respect to normal donors.

No relationship was found between the events studied (CD25 and HLA-DR expressions, changes of glycosylation) and any clinical or immunological data.

## Discussion

In SS the abnormal regulation of the immune system is partially affected by the cytokine network, including sIL-2R (5). IL-2R is considered to be an early marker of lymphocytic activation since its presence in activated cells has been demonstrated and an anomalous expression of IL-2R in the epithelial cells of LSG proved to be highly specific in patients with SS (3, 4).

Class II histocompatibility antigens such as HLA-DR are known to play a key role in antigen presentation and in the regulation of the immune response, and SS is one of the autoimmune diseases which is highly associated with these antigens (2). The finding of its overexpression on LSG tissues suggests that altered epithelial cells may play a role in the pathogenesis of SS.

The evidence of a co-expression of CD25 and HLA-DR antigens on the LSG of our patients is consistent with a previous report indicating a high level of activation of these cells, in agreement with the induction of an autoimmune process (4). This co-expression could be explained by the evidence that IL-2R is necessary for the biological effects of IL-2, which induces the release of several lymphokines, which in turn promotes HLA-DR synthesis (28, 29).

Changes in glycosylation during chronic inflammation and autoimmune diseases have been reported (11, 12, 24). In particular, specific changes in the serum (12, 18, 30) and tear (19) glyco-

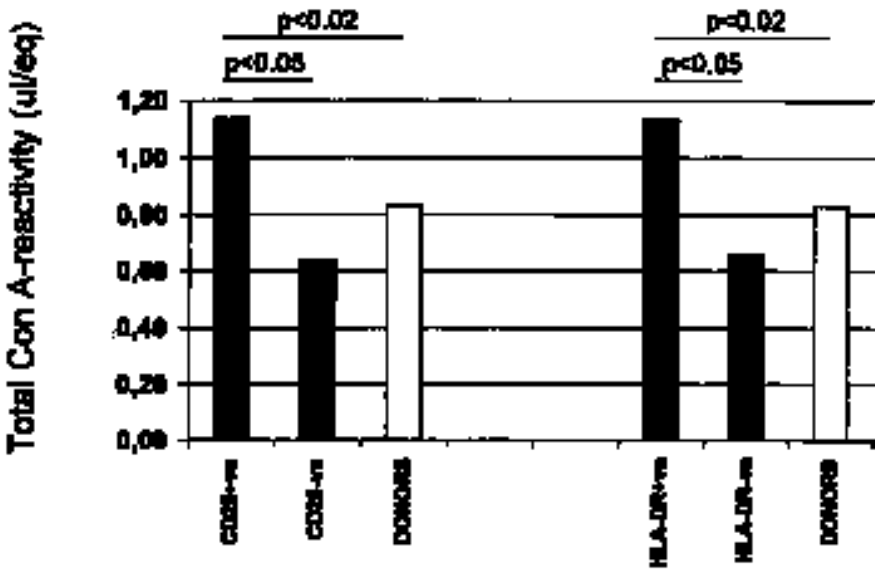



**Fig. 2.** Affinity of serum proteins for Con A,measured by ELLA,in Sjögren's syndrome patients with and without lip salivary gland positivity for anti-CD25 and anti-HLA DR monoclonal antibodies, and in normal donors.

proteins are known to occur in SS. To date we have not been able determine which specific glycidic structures are subject to alterations in SS (18), but we would speculate that variations in the N-glycan branching of selected serum proteins may be involved, as previously reported (16).

It has been suggested that glycosylation changes of tear glycoproteins could correlate with histopathological lesions at the lacrimal glandular level (19) and it is well known that lacrimal and salivary glandular involvement are both part of the same exocrinopathy in SS (31). The recent findings of Steinfeld *et al*. concerning the presence of specific mannose-binding, lectin-related structures at the LSG level in primary SS provide further information on the role played by glycoforms in this disease (20).

We have recently described that the increase in Con A reactivity occurs more frequently in the more severe disease states in SS, showing a linear correlation with serum levels of sIL-2R (18). The present study demonstrates that in SS changes in the glycosylation of serum proteins seem to be related not only to the presence of IL-2R in the sera, but also to its expression at the minor salivary gland level, as well as to the tissue co-expression of HLA-DR antigen.

It is well known that the local production of cytokines, both by mononuclear and epithelial cells, is involved in the immune-mediated destruction of exocrine glands in patients with SS (32). The immunoactivation both of the cellular infiltrate and the epithelium, detected in our study with the presence of IL-2R and HLA-DR molecules, confirms previous reports on the potential role of these molecules in the initiation and maintenance of autoimmune phenomena (2-4). The significant correlation of these immunohistological findings with changes in the glycosylation of serum proteins is of great interest for a complete assessment of the disease.

Of course, HLA-DR and CD25 expression on LSG represents only one of the aspects of SS. In fact, SS is a complex autoimmune disorder characterized by the activation of many immunological and molecular factors, including other activation and inflammatory molecules such as cytokines and other surface antigens, expressed at the level of the SS lesion (3, 32). However our results, which link for the first time the immunohistological findings of markers of immunologic activation on LSG, such as IL-2R and HLA-DR molecules, with the increase in glycosylation of serum proteins in SS patients, suggest that in this autoimmune disease the control of protein glycosylation may be mediated by the same cellular mechanisms that are involved in tissue damage. These findings may be relevant for a better knowledge of the pathogenetic mechanisms at the basis of the glandular involvement in SS.

Further studies on the changes of Con A reactive proteins in human sera may add to our understanding of the etiology and pathophysiology of many autoimmune diseases, including SS.

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