

# Immunohistochemistry of minor salivary gland biopsy specimens from patients with Sjögren's syndrome with and without hepatitis C virus infection

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## Abstract

**Objectives**—To characterise phenotypically the minor salivary glands of patients with clinical and histological features of Sjögren's syndrome (SS) infected with hepatitis C virus (HCV).

**Patients and Methods**—75 consecutive patients with SS (31 primary SS, 44 secondary SS) diagnosed by preliminary European classification criteria. The presence of anti-HCV antibodies was detected by commercial third generation ELISA and by a second generation immunoblot assay. Presence of HCV genome in serum was determined by polymerase chain reaction analysis. Expression of CD3, CD4, CD8, CD20, HLA-DR, and CD25 molecules in lymphocytic and epithelial cells on minor salivary glands was detected by immunohistochemical assays. Expression of interferon  $\gamma$  and interleukin 4 cytokines was determined by *in situ* hybridisation.

**Results**—Six of 31 primary SS (19%) and one of 44 secondary SS (2%) serum samples were positive for anti-HCV by ELISA. Three samples were positive, three indeterminate, and one sample corresponding to a secondary SS patient was negative by immunoblot. The three immunoblot positive serum samples were also HCV-RNA positive by PCR assay. The study of lymphocytic cells in the diffuse infiltrate of minor salivary glands showed a predominance of the CD3 lymphocytic population. A predominance of CD4 over CD8 T cells (ratio 2:1) was observed in HCV and non-HCV infected patients. The analysis of the lymphocytic focus showed that the HCV infected patients had a predominance of CD20 positive cells. Activation molecules (CD-25 and HLA-DR) were expressed in HCV and non-HCV infected patients in lymphocytic and epithelial cells, however epithelial cell expression of CD25 was low in HCV infected patients. As expected, a pronounced Th1 response was observed in the lymphocytic foci of HCV patients.

**Conclusions**—HCV infected patients may develop an autoimmune sialadenitis, similar to that described in primary SS.

Sjögren's syndrome (SS) is an autoimmune disease characterised by the presence of lymphocytic infiltration of the lachrymal and salivary glands, leading to keratoconjunctivitis sicca and xerostomia.<sup>1</sup> The aetiology of SS remains unknown although viral infections have been proposed as pathogenic agents.<sup>2</sup> Several studies have reported the association of hepatitis C virus (HCV) infection and SS.<sup>3-5</sup>

The aims of this study were to characterise phenotypically the minor salivary glands of HCV infected patients with clinical and histological features of SS.

## Methods

### PATIENTS

Seventy five consecutive patients attending the department of medicine were included in this study. SS was diagnosed using described criteria<sup>6</sup> and validated by preliminary European classification criteria.<sup>7</sup> The mean age of the patients was 54 years (range: 29-78). Thirty one patients had primary SS (mean 55 years; range: 29-78) and 44 had secondary SS (30 rheumatoid arthritis, 8 systemic lupus erythematosus, and 6 systemic sclerosis) (mean 52 years; range: 29-76). Ten patients were male and 65 were female. At the time that the study was begun no patient had clinical evidence of liver disease. No risk factors for HCV infection (intravenous drug abuse, recipient of blood products, tattoo, and health care occupation) were detected in the patients.

### IDENTIFICATION OF HCV INFECTION

The presence of anti-HCV antibodies was detected by commercial third generation ELISA assay (Ortho Raritan, NJ). Positive serums samples were tested by a second generation immunoblot assay (Deciscan, Pasteur). Presence of HCV genome in serum was determined by PCR analysis using primers corresponding to sequences in the 5' non-translated region of the viral genome. Nucleic acids were extracted from serum and tested for HCV-RNA using a commercial HCV-RNA detection kit (Amplicor, HCM TM Roche Molecular Diagnostic Systems, Branchburg, NJ).

### IMMUNOHISTOCHEMICAL ASSAYS

The CD3, CD4, CD8, CD20, HLA-DR, and CD25 monoclonal antibodies were used in this

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study. Assays were performed on sections of fresh frozen tissue samples as described elsewhere.<sup>8</sup>

#### IN SITU HYBRIDISATION

Twenty nine base pair synthetic biotin labelled oligonucleotides corresponding to interleukin 4 and interferon  $\gamma$  were purchased from R & D Systems. In situ hybridisation of paraffin wax sections was performed according to the manufacturer's instructions (R & D Systems) with some modifications: protein kinase K was used at 5  $\mu$ g/ml concentration and 400  $\mu$ g synthetic oligonucleotides were annealed at 37°C overnight, the hybridisation reaction was detected with avidin-alkaline phosphatase.<sup>9</sup>

#### STATISTICAL ANALYSIS

A Kruskal-Wallis non-parametric analysis of the semiquantitative data was used to assess the significance of the immunohistochemical results. A p value of < 0.05 was considered significant.

#### Results

Seventy five serum samples were first analysed for anti-HCV antibodies. Six of 31 primary SS (19%) and one of 44 secondary SS (2%) samples were positive by ELISA. As false positives have been detected by this technique a second generation immunoblot analysis was performed. From the seven serum samples positive for HCV antibodies by ELISA three tested positive for antibodies to HCV by immunoblot, three samples were indeterminate, and the serum corresponding to the secondary SS patient was negative. The three positive samples recognised four or three of the four antigens with the highest intensity against C2. The three patients with indeterminate immunoblot showed reactivity with C22.3 protein. The three immunoblot positive samples were also HCV-RNA positive by PCR assay. Ten per cent of our group of patients with primary SS therefore had viraemia for HCV.

A slight increase of the alanine aminotransferase activities (70, 62, and 84 UI/ml (normal value < 30 UI/ml)) was observed in the three patients with HCV infection and two of them also had polyclonal hypergammaglobulinaemia. None of these patients had anti-SSA (Ro) antibodies. However all of them had cryoglobulinaemia but with no associated vasculitis. In addition, the HCV infected patients had no antinuclear nor anti-LKM1 antibodies. All non-HCV infected patients had normal alanine aminotransferase values.

Thirty six minor salivary gland biopsy specimens were available for the immunohistochemical study, to analyse the expression of lymphocytic markers and activation molecules in diffuse lymphocytic infiltrate, focal lymphocytic infiltrate, and epithelial cells. The patients were divided into groups: group A comprised infected HCV patients (n=3), group B consisted of the patients with serum sample indeterminate by immunoblot analysis but negative for HCV-PCR detection (n=3) (group considered non-HCV-infected), group C comprised patients with primary SS but not HCV infection (n=15), and group D included patients with secondary SS but not infected with HCV (n=15). The immunohistochemical data were grouped in a semiquantitative scale and analysed by a non-parametric Kruskal-Wallis analysis (table 1).

#### LYMPHOCYTIC CELLS

The study of lymphocytic cells in the diffuse infiltrate showed a predominance for the CD3 lymphocytic population in all groups. A predominance of CD4 over CD8 T cells (ratio 2:1) was seen in all groups, a trait also present in primary SS. The immunohistochemical analysis of the lymphocytic focus showed that the HCV infected patients had a predominance of CD20 positive cells (p < 0.05). Activation molecules (CD-25 and HLA-DR) were expressed in both groups and no differences were detected.

#### EPITHELIAL CELLS

In epithelial cells HLA-DR and CD25 were expressed in the HCV and non-HCV infected patients, but the CD25 were lower in HCV infected patients (table 1).

A detailed analysis of the T helper subpopulation was performed by in situ hybridisation in the three HCV infected patients. For this analysis interferon  $\gamma$  and interleukin 4 cytokines were assessed as markers of Th1 and Th2 responses, respectively. As expected, a pronounced Th1 response was observed in the lymphocytic focus of HCV patients. The study of the diffuse infiltration showed almost equal populations of Th1 and Th2 cells in these patients.

#### Discussion

Our group of primary SS patients showed a 19% prevalence of anti-HCV antibodies by third generation ELISA while only 10% of the patients had HCV viraemia. Several studies have considered this question and detected prevalences ranging from 0–75%.<sup>4,5</sup> Discrepancies among the

Table 1 Phenotypical characterisation of the diffused, focal lymphocytic infiltrate, and epithelial cells in salivary glands of Sjögren's syndrome patients with and without hepatitis C virus infection

	Primary SS		Secondary SS		p Value
	Group A infected HVC (n=3)	Group B ELISA HVC indeterminate (n=3)	Group C not HVC infected (n=15)	Group D not HVC infected (n=15)	
<i>Diffuse lymphocytic infiltrate</i>					
CD3	+++	+++	+++	+++	NS
CD4	++	++	++	++	NS
CD8	++	++	++	++	NS
CD20	+	++	+	++	NS
CD25	+	+	+	++	NS
HLA-DR	++	++	++	++	NS
<i>Lymphocytic focus</i>					
Mean score*	1.00	1.6	2.6	0.9	
CD3	+	++	+++	++	NS
CD20	+++	++	+	+	<0.05
CD25	+	+	+	+	NS
HLA-DR	+	++	++	++	NS
<i>Epithelial cells</i>					
CD25	+	+	++	++	NS
HLA-DR	++	++	++	++	NS

\*Daniels's score.<sup>15</sup>

SS = Sjögren's syndrome, HVC = hepatitis virus C, + = 5–25% positive cells, ++ = 25–50% positive cells, +++ = 50–75% positive cell.

studies could be caused by: (1) different clinical criteria for the diagnosis of SS, (2) analysis of anti-HCV antibodies in serum, which can lead to false positive results because of hyperglobulinaemia or gammaglobulin treatment as noted by others<sup>4</sup> and, (3) differences in the incidence of HCV among different populations.

It is of interest to note the presence of HCV infection exclusively in primary SS patients. This result could be attributed (1) to a pathogenic role of HCV in primary SS, as has been previously suggested with other viral agents,<sup>2</sup> or (2) to the presence of a sicca syndrome similar to primary SS leading to the misclassification of asymptomatic HCV infected patients as primary SS.<sup>5</sup>

Morphological examination of the salivary glands of our HCV patients showed similar traits to primary SS. There are strikingly similar nodular patterns of lymphocyte infiltrate in salivary glands and in liver.<sup>10</sup> Haddad *et al*<sup>9</sup> reported a 57% prevalence of focal lymphocytic sialadenitis suggestive of SS in 28 patients with chronic hepatitis C. Pawlotsky *et al*<sup>11</sup> described a 14% prevalence of focal sialadenitis in 49 patients with HCV. Pirisi *et al*<sup>12</sup> described that a mild sialadenitis is a common finding in patients with HCV infection.

A broader analysis of the immunohistochemical data of minor salivary glands showed that HCV infected patients had similar behaviour to SS patients. The analysis of the three HCV infected patients showed a predominance of B over T cells in the lymphocytic focus and a pronounced expression of T lymphocytes in the inflammatory infiltrate. These findings have also been reported in patients infected with HIV.<sup>13</sup>

Patients infected with HCV expressed activation molecules (CD25 and HLA-DR) in the inflammatory infiltrate of salivary glands. Nevertheless, the percentage of lymphocytes expressing CD25 was low, in contrast with the percentage of lymphocytes expressing HLA-DR. The coexpression of class II and interleukin 2R molecules indicates a high level of activation of these cells. The presence of activated CD25+ lymphocytes in salivary glands of patients with HCV suggests possible immunological changes in these glands, an autoimmune sialadenitis. Infected patients expressed low CD25 (<10% of cells) and

HLA-DR in epithelial cells. In a previous report we described CD25 expression in the epithelia as a trait of SS patients.<sup>14</sup> Whether the sialadenitis observed in HCV infected patients is a result of the replication of HCV in salivary gland tissue or whether it is the result of a local immunological process triggered by HCV remains to be determined.

In conclusion, HCV infected patients could develop clinical and immunohistochemical patterns of salivary gland disease similar to that seen in primary SS patients.

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