T lymphocyte activation state in the minor salivary glands of patients with Sjögren's syndrome

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SUMMARY Local lymphoplasmacytoid infiltration of the diseased exocrine glands is a cardinal sign of Sjögren's syndrome (SS). The state of T lymphocyte activation present in these local infiltrations was studied by three different techniques: determination of interleukin 2 (IL2) receptor (Tac) on cell surface membrane; autoradiography combined with immunoperoxidase staining of T cell epitopes; and electron microscopic analysis of the lymphoblast subclasses. Although 64 (SEM 4)% of the local inflammatory cells expressed Ia antigen, only 4 (SEM 1)% of them displayed the T cell activation antigen Tac. Autoradiography-immunoperoxidase double labelling showed that less than 1% of all T cells in situ were [³H]thymidine incorporating blasts. This finding suggests that although T lymphocyte is the dominant cell in situ, only a few of these cells have passed the G₀/G₁ interphase, and even fewer have been pushed to the S phase of the cell cycle by IL2. Transmission electron microscopy showed that few T blasts were present, even though there were many plasma cells. This result further confirms the impression that only a minor T cell subpopulation in situ is blast transformed despite the fact that many of the local T lymphocytes in the diseased salivary glands in SS are Ia positive.

Sjögren's syndrome (SS) is an autoimmune exocrinopathy clinically characterised by xerostomia and keratoconjunctivitis sicca.^{1–2} SS is characterised by lymphocytoid cell infiltration of the lachrymal and salivary glands^{3–5}; therefore the composition of this infiltration has been analysed in many cytoadherence, histochemical, and immunohistological studies.^{6–13} The presence of B lymphocytes and plasma cells in situ has been well recorded.^{7–10} T lymphocytes, however, especially those belonging to the T4⁺ inducer/helper subset, seem to be the predominant cells in situ.^{11–13} In this work we studied the activation state of T lymphocytes in situ in the diseased glands in SS.

The cardinal feature of SS is a chronic mononuclear cell infiltration of the exocrine glands. Many of the local lymphoid cells are of B cell origin and there is much evidence indicating active B cell involvement in the pathogenesis of SS.^{7–10} This

includes hypergammaglobulinaemia, autoantibodies, demonstration of plasma cells in situ, an active immunoglobulin and rheumatoid factor synthesis in diseased glandular tissue, and the development of B cell derived malignancies in SS.¹⁴ In most patients with SS, however, T lymphocyte is the predominant immune inflammatory cell in the diseased salivary glands.^{11–13} There is little information on the state of activation of this lymphocyte subset in situ. We therefore studied the state of T cell activation in the labial salivary glands of patients with SS by three different techniques to assess Tac expression, lymphocyte ultramorphology, and DNA synthesis.

Patients and methods

PATIENTS AND BIOPSIES

Clinical characteristics of the patients studied are given in Table 1. In addition, labial salivary glands were also obtained from four patients without SS (patients 9–12; Table 1) and without any inflammatory systemic or local inflammatory disease.

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Table 1 Clinical data of the patients studied

Patient No	Age	Sex	Diagnosis	Therapy	Schirmer's test	Focus score
1	31	F	1°SS	None	4/5	3.5
2	41	F	1°SS	None	3/6	1
3	53	F	1°SS	None	3/0	9
4	28	F	2°SS/RA	NSAID	5/2	2
5	54	F	2°SS/PMR	Prednisone	0/0	2
6	67	F	1°SS	None	5/3	1.7
7	30	F	1°SS	None	0/0	4.2
8	56	F	1°SS	None	1/0	4.4
9	32	M	Fibrositis	None	19/22	0
10	39	F	Iatrogenic	None	0/0	0
11	74	F	Iatrogenic	None	ND	0.3
12	46	F	Psychogenic	None	20/24	0

1°SS=primary Sjögren's syndrome; 2°SS=secondary Sjögren's syndrome; RA=rheumatoid arthritis; PMR=polymyalgia rheumatica; NSAID=non-steroidal anti-inflammatory drug.

Six labial salivary glands were removed from each patient through a 2 cm long horizontal incision on the inside of the lower lip 1 cm from the vermilion border to the right of the midline. The local anaesthesia was 1.8 ml of Citanest-Octapressin.

Immediately after removal two glands processed for transmission electron microscopy were fixed with cold (4°C) 1.5% phosphate buffered glutaraldehyde for one hour, washed, then postfixed in cold 1.5% osmium acid for one hour. After alcohol dehydration the specimens were treated twice with propylene oxide and embedded in Epon 812. Two glands were immediately embedded in OCT compound (Lab-Tek Products, Division Miles Laboratories, Elkhart, IN), snap frozen, and stored at -20°C for immunohistochemical lymphocyte activation marker profile analysis. Immediately after removal two glands processed for autoradiography-avidin-biotin-peroxidase complex (ABC) double labelling were put into RPMI 1640 solution containing 111 kBq/ml or [³H]thymidine (185

MBq/mmol; Radiochemical Centre, Amersham, UK) and 0.5 mg/ml of thymidine phosphorylase inhibitor 5-nitrouacil, incubated at 37°C for two hours, and snap frozen in OCT.

HISTOCHEMISTRY

Cryostat sections 6 µm thick were prepared and fixed in cold (4°C) acetone for 5 minutes and then prepared for immunostaining. The sections were washed in phosphate buffered saline (0.1 M, pH 7.4) and stained with monoclonal antibodies using the ABC method introduced by Hsu *et al.*¹⁵ Technical details and staining and slide controls have been described in detail elsewhere.¹⁶ The preparations were studied with several monoclonal antibodies given in Table 2. As a further control we used inappropriate mouse IgG myeloma protein NS1 from P3×63NS1 line (Cappel Laboratories, Cochranville, PA) instead of the specific primary antibodies. Exogenous, peroxidase positive, specifically stained cells were brown and thus readily

Table 2 Characteristics of monoclonal antibodies used in this study

Monoclonal antibody	Specificity	Reference
OKT11	Total T cells	17
OKT4	Helper/inducer T cells	18
OKT8	Suppressor/cytotoxic T cells	19
OKM1	Monocytes, null cells, granulocytes	20
pan-B	B cells	21
OKIa	Activated T cells, B cells, monocytes	22
Tac	Activated lymphocytes (T cell growth factor receptor)	23, 24
OKT9	Activated lymphocytes (transferrin receptor early haematopoietic stem cells)	25
4F2	Activated lymphocytes, monocytes	26

Fig. 1 Autoradiography-avidin-biotin-peroxidase complex (ABC) double labelling of T8⁺ cells. [³H]Thymidine labelled blast cells visualised with autoradiography contain black grains over the nucleus. *

distinguishable from unstained cells under a light microscope. The cells analysed by light microscopy were counted from 6 µm thick haematoxylin counterstained tissue sections with an ocular counting square (20 squares × 20 squares) and an oil immersion objective (×1000 magnification). Standard error of the mean was used to expression dispersion, and the significance of the differences between mean values was determined with the Mann-Whitney test.

AUTORADIOGRAPHY-ABC DOUBLE LABELLING

After incorporation of [³H]thymidine and ABC staining some OKT4, OKT8, and Tac stained slides were further processed for autoradiography. They were dipped in NTB2 nuclear track emulsion (Eastman Kodak Co, Rochester, NY), exposed in darkness for 12 days, developed (Developer D-76; Eastman Kodak) for four minutes, and fixed (Rapid Fix; Eastman Kodak) before mounting.²⁷ Cells were

Fig. 2 Activation marker profile assay shows that the lymphocyte activation state in minor salivary glands differs between patients with Sjögren's syndrome and healthy controls. **p*<0.01, Mann-Whitney test.

regarded as [³H]thymidine labelled when they contained numerous grains over the cell nucleus (Fig. 1). The results of autoradiography-ABC double labelling are given as a percentage of all [³H]thymidine incorporating blasts.

TRANSMISSION ELECTRON MICROSCOPY
Ultrathin sections prepared from specimens embedded in Epon were heated to 60°C, after which the sections were stained with uranyl acetate and lead citrate for the electron microscopic study (Jeal CX-100 transmission electron microscope). Ultramorphologically, cells were regarded as lymphoblasts if they were greater than 10 µm in diameter and if the nucleus: cytoplasm ratio was less than 0.5; cells were classified as T blasts and plasma cells according to the criteria of Ishikawa and Ziff.²⁸

Results

STAINING CONTROLS

The staining and slide controls indicated the speci-

Table 3 The intensity, but not the composition, of the cellular infiltrates differs in minor salivary glands of patients with Sjögren's syndrome compared with those of healthy controls

Source of biopsy specimen	Focus score	All inflammatory cells (%)				
		T11	T4	T8	MI	B
Patients with SS	3.5 (0.9)†	67 (4)	45 (3)	21 (2)	7 (1)	12 (3)
Controls	0.1 (0.1)	53 (3)	37 (2)	13 (1)	6 (1)	8 (3)
p (Mann-Whitney's U test)	*	NS	NS	NS	NS	NS

**p*<0.01; NS=not significant.
†Values are mean (SEM).

ficity of the staining and showed that the inhibition of endogenous peroxidase was successful. Staining for endogenous peroxidase alone showed that the proportion of macrophages and monocytes was less than 10% of all inflammatory cells in situ in labial salivary glands. T4⁺ inducer/helper T lymphocytes were the predominant cells (Table 3). Although there was a more intense cellular inflammation in the patients with SS than in those without focal sialadenitis, the composition of the sparse diffuse infiltrates was similar in both (Table 3).

ACTIVATION MARKER PROFILE ASSAY

The proportions of Ia⁺, Tac⁺, T9⁺, and 4F2⁺ cells were higher in the diseased salivary glands than in normal salivary glands (Fig. 2). The proportion of Ia⁺ and 4F2⁺ cells was higher than that of Tac⁺ and T9⁺ cells both in patients with SS and controls (Fig. 2).

AUTORADIOGRAPHY-ABC DOUBLE LABELLING

The labelling index of the inflammatory mononuclear cells in situ in the salivary glands of patients with SS was less than 1%. Most of the [³H]thymidine incorporating blast cells belonged to the T4⁺ subset (69 (SEM 3)%), whereas only 26 (2)% of the [³H]thymidine incorporating blasts in situ were from the T8⁺ subset. The T4/T8 ratio, calculated from the proportion of the respective T cell subsets in all the

inflammatory mononuclear cells, was 2.3 (SEM 0.2). The ratio was surprisingly similar to the activated T4/T8 ratio calculated from the [³H]thymidine incorporating blast cell fraction (2.3 (0.2) v 2.7 (0.3)) (Fig. 3). Normal glands contained hardly any [³H]thymidine incorporating blasts.

TRANSMISSION ELECTRON MICROASSAY

Transmission electron microscopy showed only occasional T blasts (less than 1% of all inflammatory mononuclear cells in situ) in the specimens from patients with SS. Plasma cells were found peripherally to the lymphocyte foci and between the glandular acini, and they formed about 10–15% of all inflammatory mononuclear cells in situ. In the control slides normal salivary glands contained hardly any T blasts or plasma cells.

Discussion

The active involvement of T lymphocytes in the local pathogenetic mechanisms in SS has been suggested by higher percentages of Ia⁺ lymphocytes in salivary glands than those in peripheral blood lymphocytes of the same patients.¹¹ We also detected many Ia⁺ cells in situ, but it should be remembered that many other cells—such as B lymphocytes, plasma cells, monocytes, macrophages, dendritic cells, and even some resident cells—may display Ia. Surprisingly, immunohistochemical findings showed low Tac expression, confirming the earlier findings of Fox *et al.*²⁹ Furthermore, because interleukin 2 receptor has recently been reported on B cells and macrophages,²⁴ it is impossible, in the absence of double marker studies, to state that all interleukin 2 receptor bearing cells are T cells. The low percentage of IL2 receptor carrying cells in situ, however, might have been due to the blocking of immunoreactive epitopes by receptor bound IL2. This was refuted by experiments in which we used [³H]thymidine to assess the proportion of T cells that synthesised DNA in the diseased salivary glands of patients with SS²⁷; less than 1% of all local lymphocytes carrying T cell markers were [³H]thymidine incorporating blasts.

Resting lymphocytes change morphologically upon activation. Furthermore, plasma blasts and plasma cells show ultramorphological characteristics distinct from those of T cell derived activated blasts. Our electron microscopic findings on the rarity of lymphoblasts with T cell characteristics, in contrast with those with plasma blast/plasma cell characteristics, strengthen the conclusion based on the immunohistochemical findings for Tac expression and on the autoradiographical findings for DNA synthesis.

Fig. 3 Comparison of the immunoregulatory T4/T8 ratios calculated for all inflammatory mononuclear cells and for the [³H]thymidine incorporating blast cell fraction. Although the latter comprised only a minor fraction (labelling index about 1%) of all the inflammatory mononuclear cells, there was no significant difference between the total and activated T4/T8 ratios. Ratios are given as mean (SEM).

Active B cell involvement in the aetiopathogenesis of SS is amply recorded, but recent observations using monoclonal antibodies to detect lymphocytes with T cell phenotype have shown this to be the predominant immune inflammatory cell in situ. According to our findings, based on observations made with three different assays, only a minor fraction of these T cells, though Ia positive, are blast transformed. This may be related to the chronicity of the disease and does not necessarily imply that most of the local T lymphocytes are innocent bystanders. In any case, T lymphocyte activation and blast transformation seem to be more extensive in glandular tissue in SS than in normal control glands.

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