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Expression of rheumatoid factor associated cross-reactive idiotopes by glandular B cells in Sjögren's syndrome

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SUMMARY

B cell expression of the germline gene-encoded, kappa IIIb-associated, rheumatoid factor (RF) cross-reactive idiotope (CRI) 17-109 and three V_HI associated RF CRIs (G6, G8, H1) was investigated immunocytochemically in labial salivary glands from nine patients with primary and six with secondary Sjögren's syndrome, and in inflamed submandibular salivary glands from 10 patients with no history of connective tissue disease. Expression of CRIs by B cell infiltrates in labial glands from patients with primary and secondary Sjögren's syndrome were similar. Lymphoid infiltrates of labial glands from Sjögren's syndrome patients contained a higher proportion of kappa III$^+$ cells reactive for the kappa IIIb-associated 17–109 idiotope ($P < 0{\cdot}01$) and larger G6 ($P < 0{\cdot}02$) and H1 ($P < 0{\cdot}01$) positive B cell populations than those within inflamed submandibular salivary glands. Furthermore, in labial glands there was a significant correlation between numbers of 17-109 and G6 idiotope reactive cells ($r = 0{\cdot}61$; $P < 0{\cdot}02$), reflecting the known association between these H and L chain CRIs in RF IgM paraproteins. These results indicate that B cells bearing both V_KIII and V_HI-associated CRI are increased in the glandular infiltrates in Sjögren's syndrome and support the idea that this condition is associated with proliferation of immature B cell clones retaining germ-line V genes.

Keywords Sjögren's syndrome rheumatoid factors cross-reactive idiotopes B cells salivary glands

INTRODUCTION

Sjögren's syndrome is an autoimmune disease characterized by oral and ocular dryness, focal lymphoid infiltration of the exocrine glands and presence of circulating autoantibodies including rheumatoid factor (RF). Patients with the disease are known to have an increased risk of developing B cell lymphoproliferative disorders, such as monoclonal gammopathies and non-Hodgkin's lymphoma (Talal & Bunim, 1964; Kassan *et al.*, 1978). More recently it has been shown that B cell lines can be established without the use of exogenous stimulation from peripheral blood lymphocytes of patients with primary Sjögren's syndrome (Takei *et al.*, 1989; Miyasaka *et al.*, 1989). These cell lines appear to express Epstein–Barr virus (EBV) antigens (EBNA) and produce B cell growth factor spontaneously. Thus, the disordered B cell proliferation seen in this condition could be due to *in vivo* transformation, possibly by EBV, together with inappropriate lymphokine production promoting the growth of other B cells, including autoreactive B cells. This latter possibility could account for the general autoantibody profile associated with Sjögren's syndrome and expansion of specific autoantibody-producing clones, as detected by increased expression of certain RF idiotypes (Fox *et al.*, 1986b; Sugai *et al.*, 1989; Venables *et al.*, 1989a). The concept that Sjögren's syndrome is associated with disordered B cell regulation has been supported, at the local level, by studies on salivary gland lymphoid infiltrates. Recent reports indicate possible increased glandular B cell expression of the germline gene-encoded, kappa IIIb-associated, RF cross-reacting idiotope (CRI) 17-109 (Fox *et al.*, 1986*b*), presence of a detectable CD5-positive B cell population (Youinoui *et al.*, 1988) and significantly increased CD4$^+$ CD45RO$^+$ T (primed, memory or helper/inducer): CD4$^+$ CD45RA$^+$ T (virgin, naive or suppressor/inducer) ratios associated with B cell foci of affected glands (Matthews *et al.*, 1989). The possible involvement of a restricted subset of B cells in the local aetiopathology of Sjögren's syndrome prompted us to investigate the expression of 17-109 and three heavy chain associated RF CRIs (G6, G8 & H1) (Mageed *et al.*, 1986; Mageed, Carson & Jefferis, 1988) by lymphocytes infiltrating labial salivary glands of patients with this condition. Our data indicate preferential glandular B cell expression of heavy and light chain germline or minimally

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mutated germ line genes encoding RF activity in both primary and secondary Sjögren's syndrome.

SUBJECTS AND METHODS

Patients and tissues

Labial glands from 12 patients with primary Sjögren's syndrome, 14 with secondary Sjögren's syndrome associated with rheumatoid arthritis, and samples of submandibular salivary glands ($n = 14$) from patients with no history of connective tissue disease, were snap-frozen and stored in liquid nitrogen. A portion of each specimen was also fixed on neutral buffered formalin and processed for routine diagnostic histological analysis. Labial gland biopsy specimens consisted of 6–10 salivary gland lobules; 2–5 lobules were snap-frozen and 4–5 lobules were fixed and wax-embedded. Patients with Sjögren's syndrome were referred for labial gland biopsy after full investigation for connective tissue disease. They fulfilled at least three of the four diagnostic criteria proposed by Fox *et al.* (1986c) and all had clinical evidence of xerostomia, reduced whole saliva flow and/or stimulated parotid saliva flow ($< 0{\cdot}3$ ml/min), circulating autoantibodies, and focal lymphocytic infiltrates within minor glands (> 1 focus/4 mm^2). All patients with rheumatoid arthritis were seropositive for RF and fulfilled the American Rheumatological Association criteria for classical or definite rheumatoid arthritis. Patients with primary Sjögren's syndrome did not meet criteria for additional connective tissue disease.

Submandibular gland tissue was obtained from 14 patients undergoing surgery for the investigation of painless swelling of the gland or because of suspected or known sialolithiasis. Only submandibular salivary gland tissue with a histological diagnosis of non-specific sialadenitis ($n = 10$), usually associated with salivary duct calculi ($n = 7$), was investigated immunocytochemically. Histologically the glands showed diffuse inflammatory cell infiltration, duct dilatation, acinar atrophy and replacement fibrosis. The characteristic features of benign lymphoepithelial lesion, usually associated with Sjögren's syndrome, were absent.

Immunocytochemistry and evaluation of sections.

Acetone fixed, 5-μm serial sections were stained by a three-layer double-conjugate immunoperoxidase technique (Matthews *et al.*, 1988). Table 1 lists the monoclonal antibodies together with their working dilutions and specificity.

Monoclonal antibody 17-109 is reactive with a RF sequence-dependent CRI expressed by light chains of the V_κIIIb subsubgroup from RF of the Wa idiotypic group (Carson & Fong, 1983; Mageed *et al.*, 1988). G6 is reactive with a sequence-dependent V_HI associated CRI frequently but not exclusively expressed by IgM RF paraproteins containing light chains reactive for 17-109 (Crowley *et al.*, 1988; Mageed *et al.*, 1988). Monoclonal antibody H1 recognizes a sequence-dependent V_HI CRI occasionally expressed by IgM RF paraproteins reactive with G6 (Mageed *et al.*, 1988). G8 is reactive with a conformation-dependent V_HI-associated CRI present on many but not all IgM RF paraproteins reactive for G6 (Mageed *et al.*, 1986; Mageed, Goodall & Jefferis, 1990). B cells, identified immunocytochemically by staining with the L26 monoclonal antibody (Table 1), were quantified in sections of each specimen using a Seescan Prism TV image analyser (TVIA; Seescan Imaging, Cambridge, UK). This system allows automated identification of objects (e.g. individual or groups of immunostained cells) by their grey-scale value and subsequent measurements of a variety of parameters including total number of objects, areas, perimeters and feret diameters. Because of the presence of both individual and confluent groups of B cells in glandular infiltrates direct determination of B cell numbers was not possible. Areas of immunostained B cells were therefore determined, at a magnification of $\times 200$, on non-overlapping TV monitor fields (equivalent to $0{\cdot}1$-mm^2 section area) throughout the whole of each section and subsequently converted to cell numbers using a conversion factor of 8500 immunostained lymphocytes/mm^2 stained area. This conversion factor was calculated after performing both manual counting of immunostained B cells (at $\times 400$) and TVIA determination of the area of staining (at $\times 200$) in the same $0{\cdot}5$-mm^2 area of 10 different salivary gland sections. Division of the area of immunostained B cells by number of cells counted gave a mean immunostained B cell area of $117{\cdot}3 \pm 12{\cdot}7$ μm^2 (range $101{\cdot}7$–$135{\cdot}4$ μm^2) corresponding to approximately 8500 cells/mm^2 stained area.

Only labial glands containing > 200 B cells/section and exhibiting focal lymphocytic sialadenitis ($n = 15$) and submandibular glands containing > 750 B cells/section ($n = 10$) were further investigated for idiotopes. These cut-off values were chosen after our preliminary studies suggested that few idiotope-positive cells would be detectable in salivary glands and that the T:B cell ratios were about 2:1. Thus 200 B cells would correspond to about 600 lymphocytes, the minimum number investigated by Fox *et al.*, (1986b) when investigating 17-109 expression in labial glands. Essentially, seven serial sections of each gland were stained, in order, for kappa III light chains, 17-109, G6, B cells (L26), T cells (Leu4), G8 and H1. Ratios of T cells to B cells were calculated for each specimen and for individual foci within labial glands after determination of the areas of immunocytochemically stained T and B cell populations using TVIA. Numbers of kappa III and idiotope positive cells were counted manually throughout the whole of each section with the aid of an ocular grid using a $\times 40$ objective and $\times 10$ oculars. The cell counts were then converted to a percentage of the number of B cells present thus allowing for the differences in lymphocytic infiltration between specimens.

Table 1. Specificity, source and dilutions of monoclonal antibodies

Antibody	Dilution	Specificity	Source/reference
L26	1/100	B cells	Dako
Leu 4	1/200	CD3,	T cells Becton Dickinson
C7	1/4000	kappa III	Mageed *et al.* (1986, 1988)
17-109*	1/1600	V_κ IIIb	CRI Carson & Fong, 1983; Fox *et al.* (1986b)
G6†	1/32000	V_H1 CRI	Mageed *et al.* (1986, 1988, 1990)
H1	1/16000	V_H1 CRI	
G8	1/16000	V_H1 CRI	

Peroxidase-conjugated rabbit anti-mouse immunoglobulin and swine anti-rabbit immunoglobulin were purchased from Dako and used diluted 1/100 in buffer containing 20% normal human **AB** serum.

* Idiotope encoded by germline gene HumKv 325.

† Idiotope encoded by germline gene 51p1 or minimally mutated product.

17-109-positive cells were also expressed as a percentage of kappa III-positive cells detected in each specimen.

Statistical analysis

Data were analysed using Minitab statistical software (release 7) and significant differences determined by the Mann–Whitney *U*-test.

RESULTS

As expected, all specimens contained both B and T cell infiltrates (Fig. 1). The mean T:B cell ratios for primary ($2{\cdot}3 \pm 1{\cdot}9$:1) and secondary ($2{\cdot}5 \pm 2{\cdot}3$:1) Sjögren's glands and submandibular salivary glands ($3{\cdot}3 \pm 2{\cdot}4$:1) were not significantly different from each other and ranged between 0·5:1 and 8·5:1. The 15 labial gland specimens contained a total of 53 lymphocytic foci. T:B cell ratios in foci, within and between specimens, showed great variation and ranged between 0·3 and 17:1, giving a mean T:B ratio for foci of $3{\cdot}0 \pm 3{\cdot}6$:1.

B cell, kappa III light chain and idiotope positive cell counts, together with the counts expressed as a percentage of the B cell population within each specimen, are shown in Table 2. All specimens contained kappa III-positive cells which accounted for between 0·4% and 33·7% of immunocytochemically detectable B cells. Although the mean kappa III-positive cell population in submandibular glands (9·4%) was greater than twice that detected in labial glands (4·1%) this difference was not significant at the 5% level due to the large within group variation. Subjectively, the kappa III-positive cells in submandibular glands appeared to be predominantly plasma cells with little tendency to be associated with areas of inflammation. By contrast, labial glands exhibited a mixed population of cytoplasmically stained plasma cells/blasts, distributed throughout the specimens, and lymphocytes showing weak membrane staining for kappa III light chains, in areas showing focal infiltrates. This pattern of staining was also exhibited by idiotope positive cells (Fig. 1).

Kappa III- and idiotope-positive cell populations were similar in labial glands from patients with primary and secondary Sjögren's syndrome. Analysis of cell counts from all labial glands demonstrated a significant correlation between kappa III cell numbers and cells bearing the kappa IIIb-associated RF idiotope 17-109 ($r = 0{\cdot}89$; $P < 0{\cdot}001$). Furthermore, a significant correlation was found between the number of cells expressing the 17-109 idiotope and those reactive for the V_HI-associated G6 idiotope ($r = 0{\cdot}61$; $P < 0{\cdot}02$) with which it is often associated in IgM RF paraproteins (Crowley *et al.*, 1988; Mageed *et al.*, 1988). By contrast there were no significant correlations between numbers of cells positive for these idiotopes within the control submandibular salivary gland group. However, as found in labial gland specimens, there was a significant correlation between kappa III and 17-109 idiotope-positive cell numbers in submandibular gland specimens ($r = 0{\cdot}79$; $P < 0{\cdot}01$).

As a percentage of the B cell population, labial glands contained significantly more G6 positive ($P < 0{\cdot}02$) and H1 positive ($P < 0{\cdot}01$) cells than submandibular glands. Although mean values for 17-109- and G8-expressing B cells in labial glands (1·4% and 0·8% respectively), were greater than twice those in submandibular glands (0·3% and 0·3%), these differences were not significant because of the large within group variation. Cells reactive for the kappa IIIb-associated 17-109 idiotope, however, accounted for a significantly greater proportion of the kappa III population in labial glands (32·6%) than in submandibular glands (3·8%; $P < 0{\cdot}01$).

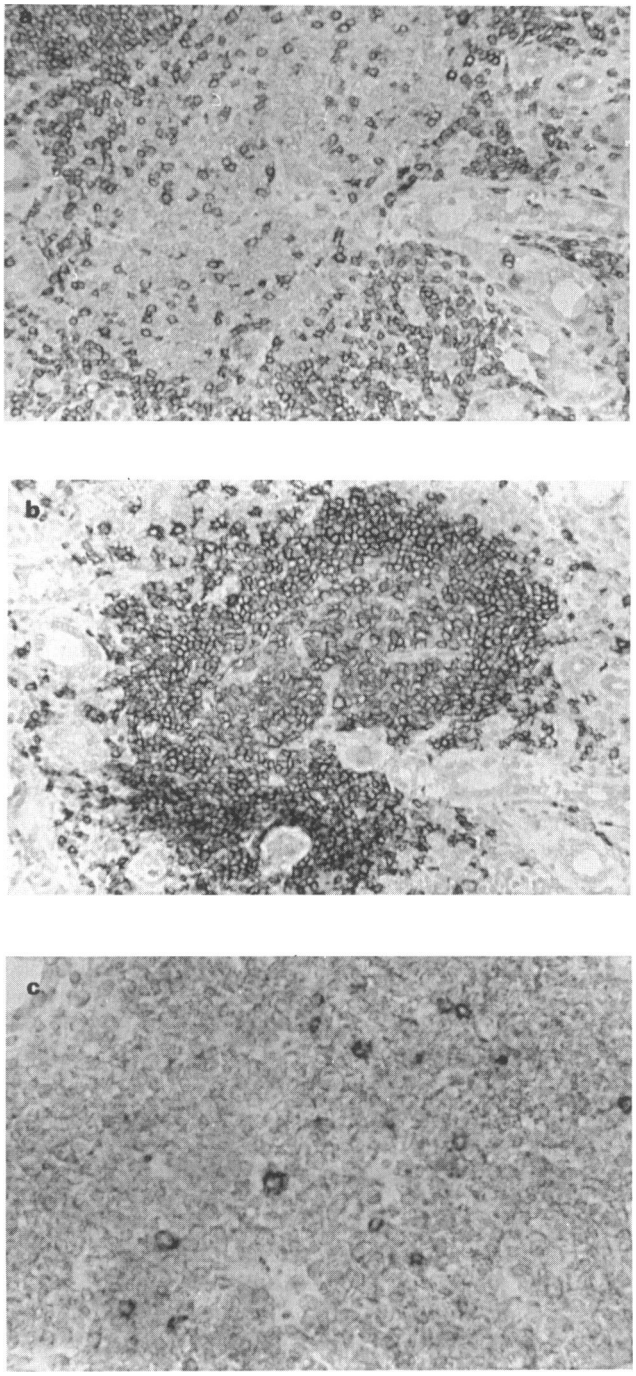

Fig. 1. A large focus of lymphocytes within a labial gland from a patient with primary Sjögren's syndrome stained for T cells (a), B cells (b) and G6 idiotope (c) Immunoperoxidase–haematoxylin. Magnification; a, b, ×160; c, ×220.

DISCUSSION

Labial salivary glands from patients with primary and secondary Sjögren's syndrome contain elevated numbers of lympho-

Table 2. Summary of kappa III- and idiotope-positive cell counts

Specimen number	B cells	KIII$^+$ and CRI$^+$ cells (% of B cells)					17-109$^+$ cells (% of KIII$^+$ cells)
		KIII	17-109	G6	G8	H1	
Labial glands							
Primary Sjögren's syndrome							
1	3750	35 (0·9)	17 (0·5)	58 (1·6)	12 (0·3)	21 (0·6)	48·6
2	1217	146 (12)	82 (6·7)	65 (5·3)	22 (1·8)	25 (2·1)	56·2
3	300	6 (2·0)	0 (0)	3 (1·0)	6 (2·0)	1 (0·3)	0·0
4	1667	25 (1·5)	2 (0·1)	19 (1·1)	8 (0·5)	2 (0·1)	8·0
5	3333	20 (0·6)	11 (0·3)	76 (2·3)	1 (0·03)	0 (0)	55·0
6	283	16 (5·7)	9 (3·1)	15 (5·3)	5 (1·8)	2 (0·7)	56·3
7	342	18 (5·2)	1 (0·3)	6 (1·8)	3 (0·8)	6 (1·8)	5·6
8	417	4 (0·9)	0 (0)	10 (2·3)	1 (0·2)	1 (0·2)	0·0
9	2858	71 (2·5)	44 (1·5)	52 (1·8)	1 (0·03)	2 (0·1)	62·0
Mean %		3·5	1·4	2·5*	0·8	0·6‡	32·4
(s.d.)		(3·7)	(2·3)	(1·7)	(0·8)	(0·8)	(27·8)
Secondary Sjögren's syndrome							
1	2925	13 (0·4)	12 (0·4)	33 (1·1)	6 (0·2)	24 (0·8)	92·3
2	1000	55 (5·5)	20 (2·0)	39 (3·9)	38 (3·8)	10 (1·0)	36·4
3	950	104 (11)	26 (2·7)	18 (1·9)	0 (0)	24 (2·5)	25·0
4	992	12 (1·2)	1 (0·1)	0 (0)	0 (0)	5 (0·5)	8·3
5	625	66 (10·6)	17 (2·7)	0 (0)	0 (0)	0 (0)	25·8
6	4633	49 (1·1)	5 (0·1)	11 (0·2)	1 (0·02)	12 (0·3)	10·2
Mean %		5·0	1·3	1·2	0·7	0·9§	33·0*
(s.d.)		(4·9)	(1·3)	(1·5)	(1·5)	(0·9)	(30·9)
All Sjögren's syndrome (n = 15)							
Mean %		4·1	1·4	2·0‡	0·8	0·7†	32·6†
(s.d.)		(4·1)	(1·9)	(1·7)	(1·1)	(0·8)	(28·3)
Submandibular glands							
1	780	161 (20·6)	12 (1·5)	8 (1·0)	7 (0·9)	7 (0·9)	7·5
2	6117	318 (5·2)	35 (0·6)	96 (1·6)	27 (0·4)	16 (0·3)	11·0
3	1485	159 (10·7)	0 (0)	9 (0·6)	9 (0·6)	0 (0)	0·0
4	2299	180 (7·8)	6 (0·3)	23 (1·0)	6 (0·3)	0 (0)	3·3
5	772	260 (33·7)	3 (0·4)	2 (0·3)	2 (0·3)	0 (0)	1·2
6	4623	157 (3·4)	6 (0·1)	15 (0·3)	10 (0·2)	0 (0)	3·8
7	7611	451 (5·9)	33 (0·4)	3 (0·04)	14 (0·2)	0 (0)	7·3
8	5362	183 (3·4)	4 (0·1)	0 (0)	2 (0·04)	0 (0)	2·2
9	17978	102 (0·6)	2 (0·01)	154 (0·9)	17 (0·1)	3 (0·02)	2·0
10	7536	230 (3·1)	0 (0)	31 (0·4)	0 (0)	0 (0)	0·0
Mean %		9·4	0·3	0·6	0·3	0·1	3·8
(s.d.)		(10·2)	(0·5)	(0·5)	(0·3)	(0·3)	(3·7)

Significance in Mann–Whitney *U*-test against the submandibular gland group: *$P < 0·003$; †$P < 0·01$; ‡$P < 0·02$; §$P < 0·04$.

cytes bearing H chain (G6, H1) and L chain (17-109) RF-associated CRIs. Although 17-109-positive lymphocytes have been detected in glands from patients with primary Sjögren's syndrome previously (Fox *et al.*, 1986b) this is the first study to document increased glandular expression of both heavy and light chain RF CRIs compared with diseased, control, glandular tissues.

The general predominance of G6 idiotope-bearing cells in all salivary glands, the significant correlation between the numbers of cells expressing G6 and 17-109 in glands from Sjögren's syndrome patients and the overall increase in 17-109, G6 and H1 in Sjögren's syndrome reflect the known relationships between these CRI on RF paraproteins. Thus the kappa IIIb-associated 17-109 idiotope, known to be more highly expressed on RF from Sjögren's syndrome patients (Fox *et al.*, 1986b), has been shown to be frequently associated with the V_HI-associated G6 idiotope among IgM paraproteins having RF activity (Crowley *et al.*, 1988). G8 and H1, in turn, appear to be co-expressed by subpopulations of G6 positive RF paraproteins (Mageed *et al.*, 1988, 1990). Furthermore, IgM-bearing G6 is elevated in the sera of patients with primary Sjögren's syndrome and to a lesser extent in sera of patients with infectious mononucleosis, rheumatoid arthritis and systemic lupus erythematosus (Venables *et al.*, 1989a; Shokri *et al.*, 1990a). Taken together, these

results suggest that in Sjögren's syndrome germ-line genes coding for both heavy and light chain CRIs are preferentially expressed at the systemic level by circulating immunoglobulin and, at the local level, by glandular B cells.

An unexpected finding was the low incidence of kappa III bearing cells in labial glands (4·1 ± 4·1%). V_K III is a major V_K subgroup and, theoretically, might be expected to account for about 20% of all B cells (assuming 60:40 kappa-to-lambda ratio and that V_K III accounts for about 30% of all V_K; Capra *et al.*, 1972; Kunkel *et al.*, 1974). Submandibular glands contained a higher mean percentage of kappa III-bearing cells (9·4 ± 10·2%) with great variation between specimens (range 0·6–33·7%). We believe that the variation in kappa III expression between specimens is a true reflection of variations in the glandular B cell infiltrates as preliminary data in tonsil (data not shown), using the same antisera and quantification methods, suggest that approximately 10% (range 8·5–12%; $n = 4$) of B cells bear kappa III. The reason for the lower than predicted percentage expression of kappa III-bearing cells in both salivary glands and tonsil is unknown but may relate to the specificity of the monoclonal antibody used and the availability of the kappa III epitope within intact immunoglobulin on the cell surface or within the cytoplasm of B cells.

The factors responsible for determining the levels of CRIs in Sjögren's syndrome are unknown. The observation that two of the CRIs occurring at elevated levels (17-109 and G6) are coded for by germline genes or their minimally mutated counterpart suggests that the stimulus, if extrinsic, must cause activation of restricted subsets of B cells. EBV has been suggested as a possible candidate as raised levels of the G6 idiotope-bearing IgM occur in both Sjögren's syndrome and infectious mononucleosis (Venables *et al.*, 1989a). However, the relation between EBV and Sjögren's syndrome is controversial, as both increased and decreased detection of EBV antigens and DNA have been reported within affected glands (Fox, Pearson & Vaughan, 1986a; Venables *et al.*, 1989a, 1989b; Saito *et al.*, 1989; Schuurman *et al.*, 1989; Deacon *et al.*, 1990). The idiotype profile in Sjögren's syndrome might result from intrinsic B and/or T cell defects. For example, CD5-positive B cells, known to be associated with autoantibody production (Hardy *et al.*, 1987; Casali *et al.*, 1987), are increased in patients with rheumatoid arthritis (Plater-Zyberk *et al.*, 1985; Brennan *et al.*, 1989) and Sjögren's syndrome (Youinoui *et al.*, 1988; Dauphinee, Tovar & Talal, 1988; Brennan *et al.*, 1989). Activation of this expanded B cell subpopulation, due to imbalances of sex hormone levels (Ahmed *et al.*, 1989), virus infection (Casali *et al.*, 1987) and/or regulatory T cell defects, might account for the heightened RF CRI expression. Indeed, at the local level, B cell foci within salivary glands contain CD5 positive B cells (Youinoui *et al.*, 1988) and are associated with an increased T helper/inducer (memory) cell population (Matthews *et al.*, 1989).

Although the finding of CRI positive cells in glands from Sjögren's syndrome patients might merely reflect increased numbers of such cells in the circulation, the studies of Fox *et al.*, (1986b) indicate that this is not the case and suggest that other mechanisms such as selective localization of CRI bearing cells to the salivary glands or *in situ* clonal expansion of CRI-bearing cells are responsible. Direct evidence for active lymphocyte proliferation within affected glands is lacking and we have failed to detect significant numbers of proliferating cells *in situ* using the Ki67 monoclonal antibody (data not shown). Similarly, no studies have demonstrated selective migration of CRI bearing cells to salivary glands.

The major significance of our study relates to the fact that patients with primary Sjögren's syndrome and cryoglobulinaemia are at a higher risk of developing lymphoma than patients with Sjögren's syndrome alone (Talal & Bunim, 1964) and that both the kappa IIIb-associated 17-109 and the V_HI-associated G6 CRIs are expressed on RF paraproteins from patients with cryoglobulinaemia (Mageed *et al.*, 1988) and have been detected on tumour cells and associated serum paraproteins from patients with Sjögren's syndrome and lymphoma (Fox *et al.*, 1983, 1986b; Shokri *et al.*, 1990a, 1990b). These observations support the concept that in Sjögren's syndrome there is proliferation of immature B cell clones bearing germline gene-encoded CRI (and possibly CD5) in the absence of somatic diversification which, in certain circumstances, can lead to the development of malignant lymphoma. We have demonstrated this expanded B cell population locally in both primary and secondary Sjögren's syndrome which contrasts with the low incidence of CRI-bearing cells associated with chronic antigen stimulation and inflammation within submandibular glands of patients without Sjögren's syndrome or connective tissue disease. Whether the primary Sjögren's syndrome patients exhibiting high (48·6–62%) or low (0–8%) levels of 17-109 reactive cells (Table 2), as a percentage of kappa III-positive cells, represent risk categories in respect of lymphoma development, disease subgroups, or sampling artefact due to the small numbers of B- and CRI-positive cells in the 'low' group, awaits further investigation. The possible clinical significance of monitoring glandular B cells for expression of idiotope producing clones as an early indicator of lymphoma development in Sjögren's syndrome remains to be evaluated.

Our data suggests that B cells bearing both V_KIII- and V_HI-associated CRI are increased in the glandular infiltrates in Sjögren's syndrome supporting the idea that this condition is associated with proliferation of immature B cell clones retaining germline V genes.

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