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Primed and naive helper T cells in labial glands from patients with Sjogren's syndrome

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Summary. This study has investigated the presence and distribution of B cells, T cells and T-cell subsets within labial glands of patients with primary Sjogren's syndrome ($n=9$) and secondary Sjogren's syndrome associated with rheumatoid arthritis ($n=8$) using a sequential double immunoperoxidase technique and true colour image analysis. The composition of the inflammatory infiltrates was similar in glands from both patient groups. B cells were normally present within large foci with few detected in diffuse infiltrates such that the ratio of T:B cells in foci (2.4:1) was significantly lower than in diffuse infiltrates (7.3:1; $P<0.001$). In all infiltrates helper T cells ($CD8^-$, $CD3^+$) predominated over suppressor/cytotoxic cells ($CD8^+$, $CD3^+$; 2.7:1). Analysis of primed ($CD45RA^-$, $CD45RO^+$) and naive ($CD45RA^+$, $CD45RO^-$) $CD8^-$ T cells showed that the ratio of the primed to naive subset was significantly higher in focal (4.2:1) compared to diffuse (1.5:1; $P<0.001$) areas of lymphoid infiltration. These results indicate that the focal lymphocytic infiltrates characteristic of Sjogren's syndrome contain B cells associated with a T-cell population consisting predominantly of primed $CD8^-$ helper T cells. This latter population may be responsible for upregulating glandular B-cell activity in Sjogren's syndrome.

Key words: $CD4^+$ T cells – Primed helper T cells – Naive helper T cells – Salivary glands – Sjogren's syndrome

Introduction

Previous immunocytochemical studies of labial salivary glands from patients with primary and secondary Sjogren's syndrome have demonstrated that the major cell present within the focal lymphocytic infiltrates characteristic of this disease is the $CD4^+$ T lymphocyte (Adamson et al. 1983; Lindahl et al. 1985; Matthews et al. 1986). Although several studies have investigated the functional status of B lymphocytes within affected glands in respect of their ability to synthesise and secrete autoantibodies (Anderson et al. 1972; Horsfall et al. 1989) and immunoglobulins bearing cross-reactive idiotopes associated with such antibodies (Fox et al. 1986a; Deacon et al. 1991), little is known about the functional status of the predominant $CD4^+$ T-lymphocyte population.

T lymphocytes express multiple forms of the leucocyte common antigen (CD45) which are transcribed by alternative usage of CD45 exons 4–6. $CD4^+$ T cells can be divided into two reciprocal subsets expressing either the high-molecular-weight isoforms of leucocyte common antigen (CD45RA, CD45RB) or the low-molecular-weight variant in which exons 4–6 have been spliced out (CD45RO; Thomas 1989). Functionally, $CD45R^+$ CD4 T cells, which cannot respond to soluble recall antigens, are believed to represent naive or virgin cells whereas those expressing the exonless CD45RO isoform, which can respond to recall antigens and provide help for antibody synthesis (Morimoto et al. 1985), appear to be antigen primed or memory cells. Although it is unclear whether loss of CD45R and maturation from naive ($CD45R^+$) to primed ($CD45RO^+$) T cells reflects unidirectional (Sanders et al. 1988) or reversible (Bell and Sparshott 1990) differentiation after antigenic activation, it is known that it is associated with increased expression of CD2, lymphocyte function-associated antigen 1 (LFA-1), LFA-3 and CD29 (Akbar et al. 1988; Sanders et al. 1988; Serra et al. 1988).

$CD45RO^+$ primed CD4 T cells have been suggested to be increased and predominate in the peripheral blood of patients with a variety of diseases including rheumatoid arthritis (Emery et al. 1987), systemic lupus erythematosus (Sato et al. 1987; Raziuddin et al. 1989), systemic sclerosis (Rose et al. 1985; Gustafsson et al. 1990) and Sjogren's syndrome (Sato et al. 1987). Although a similar preponderance of the $CD4^+$ primed subset ap-

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pears to occur within most lesional tissues studied (Moore et al. 1988; Modlin et al. 1988; Stein-Oakley et al. 1989; Katayama et al. 1990), the data available to date are limited because of the inherent difficulties of in situ quantification of cell subsets which are characterised by antigens (CD45RA and RO, CD29, CD4) shared by other cell types, including $CD8^+$ T cells, B cells, NK cells, macrophages, epithelial and endothelial cells.

In order to characterise the functional status of the $CD4^+$ T cells within the lymphoid infiltrates of labial salivary glands from patients with Sjogren's syndrome we have investigated the presence and distribution of $CD8^-$ T-cell subsets using a two-colour indirect immunoperoxidase technique and combinations of monoclonal antibodies allowing phenotypic identification of both the naive and primed subsets.

Materials and methods

Patients and tissues. Labial salivary gland biopsy was performed under local anaesthesia on patients with primary Sjogren's syndrome ($n=9$) and secondary Sjogren's syndrome associated with rheumatoid arthritis ($n=8$). Three to four salivary gland lobules from each specimen were snap frozen and stored in liquid nitrogen. The remainder of the tissue was fixed in neutral buffered formalin and processed to paraffin wax for routine histological analysis. All patients 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. (1986b) and all had clinical evidence of xerostomia, reduced whole saliva flow and/or stimulated parotid saliva flow (<0.3 ml/min), circulating autoantibodies, and focal lymphocytic infiltrates within minor glands (>1 focus/4 mm^2). All patients with rheumatoid arthritis were seropositive for rheumatoid factor and fulfilled the American Rheumatism Association criteria for classical or definite rheumatoid arthritis. Patients with primary Sjogren's syndrome did not meet criteria for additional connective tissue disease.

Immunocytochemistry. Serial, 5-μm-thick cryostat sections were air dried (60 min), fixed in dry acetone (30 min) and air dried (5–10 min) prior to staining by a double indirect immunoperoxidase technique. Essentially, dry sections were incubated with the first monoclonal antibody or antibody mixture (60 min; Table 1), washed in buffer (×3) and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Copenhagen, Denmark 1/100 dilution in buffer containing 10% normal human AB serum, 30 min). After washing (×3), bound peroxidase was visualised using 3,3′ diaminobenzidine reagent (5 mg DAB in 10 ml buffer plus 10 μl 30% hydrogen peroxide, 5 min), and unreacted substrate was removed by further washing (×3) prior to application and incubation with the second monoclonal antibody (60 min, Table 1). Sections were thereafter washed (×3), incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, 1/100 dilution in buffer containing 10% normal human AB serum, 30 min), washed (×3), incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako, 1/100 dilution in buffer containing 10% normal human AB serum, 30 min), washed (×3) and the bound peroxidase visualised using 4-chloro-1-naphthol reagent (10 mg in 20 ml buffer plus 10 μl 30% hydrogen peroxide, 5–10 min). After further washing sections were mounted in glycerol jelly without prior counterstaining. All procedures were performed at room temperature and TRIS-buffered saline, pH 7.6, was used for all antibody dilutions and washing steps.

The additional step of peroxidase-conjugated swine anti-rabbit immunoglobulin in the second staining run was included to increase the chloronaphthol reaction and counteract the problem of identifying small numbers of pale blue cells in an area containing large numbers of dark brown DAB-positive cells. The combinations of monoclonal antibodies used and the cell populations identified are shown in Table 1. These combinations were chosen to eliminate, as far as is practicable, inclusion of non-$CD4^+$ T cells in estimates of primed and naive subsets. The method assumes that all $CD8^-$ T cells will express CD4. The apparently simpler combinations of WR16 or UCHL1 followed by anti-CD4 to identify primed and naive cells respectively, by sequential or true double staining techniques, was rejected because of the very weak staining obtained

Table 1. Monoclonal antibody[a] combinations used to detect lymphocyte populations

No.	Antibody combinations used		Cell populations identified	
	First mAb	Second mAb	Brown cells	Blue cells
1	L26	Leu4	B cells	T cells
2	B941	Leu4	$CD8^+$ cells	$CD8^-$ T cells (helper)
3	B941 + WR16	Leu4	$CD8^+$ and/or $CD45RA^+$ cells	$CD8^-$, $CD45RA^-$ T cells (primed helper)
4	B941 + UCHL1	Leu4	$CD8^+$ and/or $CD45RO^+$ cells	$CD8^-$, $CD45RO^-$ T cells (naive helper)
5	B941 + L26 + BRC-M1/MA5	WR16	$CD8^+$ cells, B cells and Macrophages	$CD8^-$, $CD45RA^+$ T cells (naive helper)
6	B941 + L26 + BRC-M1/MA5	UCHL1	$CD8^+$ cells, B cells and Macrophages	$CD8^-$, $CD45RO^+$ T cells (primed helper)

[a] Specificity, source and dilution of antibodies:
First layer: L26 (CD20, Mason et al. 1990; Dako, Copenhagen, Denmark), 1/50; B941 (CD8, Dept. of Immunology, University of Birmingham), 1/200; WR16 (CD45RA, Moore et al. 1987), 1/200; UCHL1 (CD45RO, Dako), 1/50; BRC-M1/MA5 (Macrophages, Serotec, Kidlington, England), 1/100.
Second layer: Leu4 (CD3, Becton-Dickinson, Cowley, England), 1/100; WR16, 1/100; UCHL1, 1/25

by two different anti-CD4 antibodies (Leu3a and Dako T4) when used in the second staining step and the possibility of detecting CD4 positive macrophages (Matthews et al. 1984). Positive identification of the primed subset using anti-CD29 (4B4 and WR19) was unsuccessful because of the known reactivity of epithelium, vascular endothelium and macrophages for this antigen (Modlin et al. 1988; Moore et al. 1988). Four serial sections of all specimens were stained, in order, with antibody combinations 1, 2, 3 and 5. Three specimens were further investigated by staining another series of four serial sections for antibody combinations 5, 3, 4 and 6 (Table 1).

Controls. The following controls were performed on sections of palatine tonsil and submandibular salivary gland tissue showing inflammatory changes and the observations described apply to all antibody combinations unless stated otherwise. Omission of the first monoclonal antibody resulted in presence of blue stained cells alone which were present in similar numbers and in the same distribution as serial sections conventionally stained with the second monoclonal antibody alone. All staining runs included one set of sections in which the first monoclonal antibody/antibody mixture was replaced with buffer. As expected, omission of the first peroxidase-conjugated rabbit anti-mouse immunoglobulin layer and/or the DAB step resulted in detection of blue cells corresponding to cell populations identified by both monoclonal antibodies. Thus, with the antibody combinations used in this staining system, binding of the first monoclonal antibody by peroxidase-conjugated rabbit anti-mouse immunoglobulin followed by the DAB reaction effectively precluded binding of the second monoclonal antibody and conjugated antibodies from binding to the same cells.

Omission of the second peroxidase-conjugated rabbit anti-mouse immunoglobulin layer or both second conjugated antibody layers resulted in very pale reactivity or absence of blue cells respectively. Omission of the peroxidase-conjugated swine anti-rabbit immunoglobulin layer alone diminished the staining intensity and, in some cases, the number of blue cells detected. Inverting the order of application of monoclonal antibodies in the L26-Leu3 and B941-Leu4 combinations resulted in a reciprocal staining pattern and brown cells only, respectively. Omission of both monoclonal antibodies removed all positive reactivity whereas use of the same monoclonal antibody or combination as both first and second monoclonal antibody gave brown cells only.

Evaluation of sections. Double-stained sections were stored in the dark for a maximum of 5 days prior to evaluation. This precaution was taken because the blue chloronaphthol reaction product is subject to fading especially when left exposed to light.

Estimation of T:B and $CD8^+$:$CD8^-$ T-cell ratios was performed by area measurements of blue and brown stained cell populations within lymphocytic foci or areas showing diffuse infiltration using a Seescan Prism colour TV image analysis system (Seescan Imaging, Cambridge, UK) and microscope fitted with a $\times 25$ objective. A minimum of five foci and, when present, five areas of "diffuse" infiltration were examined per specimen. A focus was defined as a collection of 50 or more lymphocytes: so-called diffuse areas contained small foci (i.e. < 50 lymphocytes) and scattered individual lymphocytes in histologically normal parts of the specimens.

Ratios of primed to naive $CD8^-$ T cells were determined from cell counts of blue cells within the same five high power fields of focal or diffuse lymphocytic infiltrate in serial sections of each gland stained for these subsets. Ratios for all specimens were obtained from sections stained with antibody combinations 3 and 5 (Table 1). Four different memory: naive ratios were determined for the three specimens stained with the four antibody combinations 5, 3, 4 and 6. Slides were examined at $\times 400$ magnification using a microscope fitted with an eyepiece graticule. Image analysis was not used for this part of the study because of the relatively low number of naive cells and difficulties in resolving low numbers of pale blue cells against a background of dark brown cells.

Statistics. Data were analysed using the Mann-Whitney U test.

Results

All 17 labial gland specimens exhibited focal lymphocytic infiltration and 12 also contained areas of diffuse infiltration with small accumulations of lymphocytes (Fig. 1). Subjective analysis of the immunostained sections failed to reveal any differences between glands from the two patient groups. This was confirmed by determination of T:B, $CD8^-$:$CD8^+$ T-cell and memory:naive helper T-cell ratios (Table 2). For a given type of infiltrate there were no significant differences between glands from primary and secondary Sjogren's syndrome patient groups. However, both subjectively and by quantitative determination of immunostained cell ratios there were clear differences in the distributions of some cell types. Areas of diffuse lymphoid infiltration and small accumulations of lymphocytes contained few B cells and

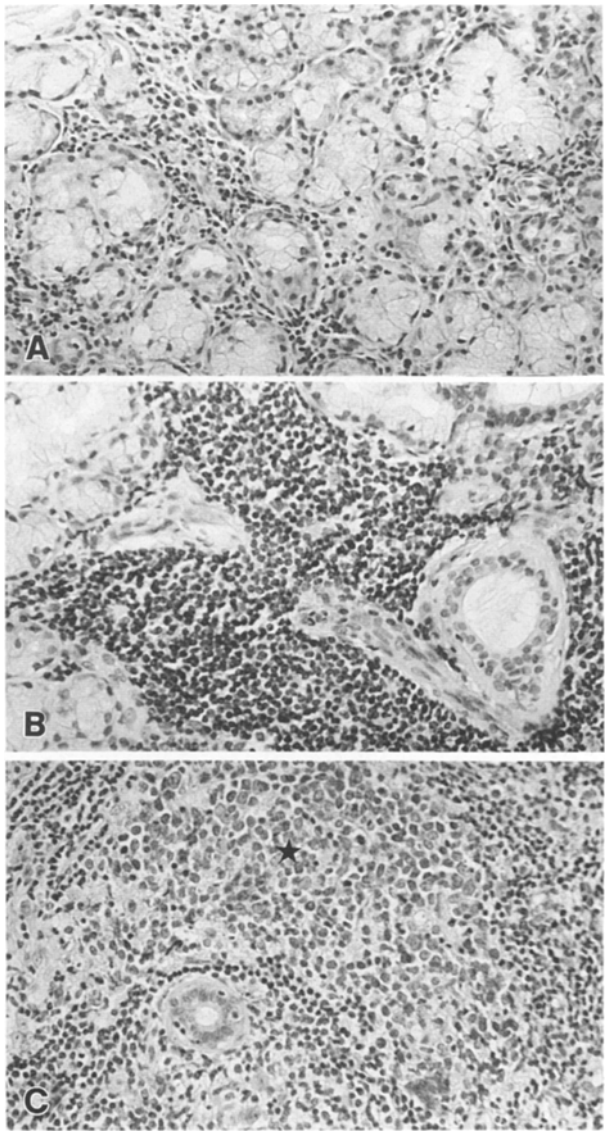

Fig. 1. A diffuse lympho-plasmacytic infiltrate (**A**) and periductal lymphocytic foci (**B, C**). The germinal centre-like structure (*) within the large lymphoid focus (**C**) corresponds to an area composed predominantly of CD20 positive B lymphocytes. Haematoxylin and eosin, $\times 200$

Table 2. Mean ratios (±SD) of cell populations within focal and diffuse areas of infiltration

Type of infiltrate	Sjogren's group	Cell ratios (range)			Mean counted/field[b] (±SD)
		T:B cells	$CD8^-$:$CD8^+$ T	Primed:naive[a]	
Focal	Primary	2.5±1.9:1	2.5±1.2:1	3.9±1.4:1	120.6±27.4
	(n=9)	(0.5–6)	(0.7–4.1)	(1.5–5.9)	35.5±11.1
	Secondary	2.4±2.0:1	2.7±1.2:1	4.5±1.6:1	103.0±30.0
	(n=8)	(1.0–7)	(0.8–4.0)	(2.0–6.9)	24.8± 5.9
	All	2.4±1.9:1	2.6±1.2:1	4.2±1.5:1	
	(n=17)	(0.5–7)	(0.7–4.1)	(1.5–6.9)	
Diffuse	Primary	7.7±5.5:1	2.8±1.3:1	1.4±0.6:1	18.9± 8.1
	(n=7)	(3–17)	(1.1–4.4)	(0.6–2.4)	15.0± 4.1
	Secondary	6.6±2.7:1	2.8±0.8:1	1.7±1.0:1	18.3± 6.1
	(n=5)	(4–11)	(1.6–3.6)	(0.7–3.4)	12.6± 3.1
	All	7.3±4.4:1	2.8±1.1:1	1.5±0.8:1	
	(n=12)	(3–17)	(1.1–4.4)	(0.6–3.4)	
Significance[c]		$P<0.001$	NS	$P<0.001$	

[a] Primed:naive helper T-cell ratios determined using serial sections stained by antibody combinations 3 and 5 (Table 1)
[b] Mean numbers of primed (upper) and naive (lower) cells counted per field
[c] Mann Whitney U test; difference between ratios determined in focal and diffuse infiltrates. There were no significant differences between primary and secondary Sjogren's syndrome for a given type of infiltrate. NS, not significant

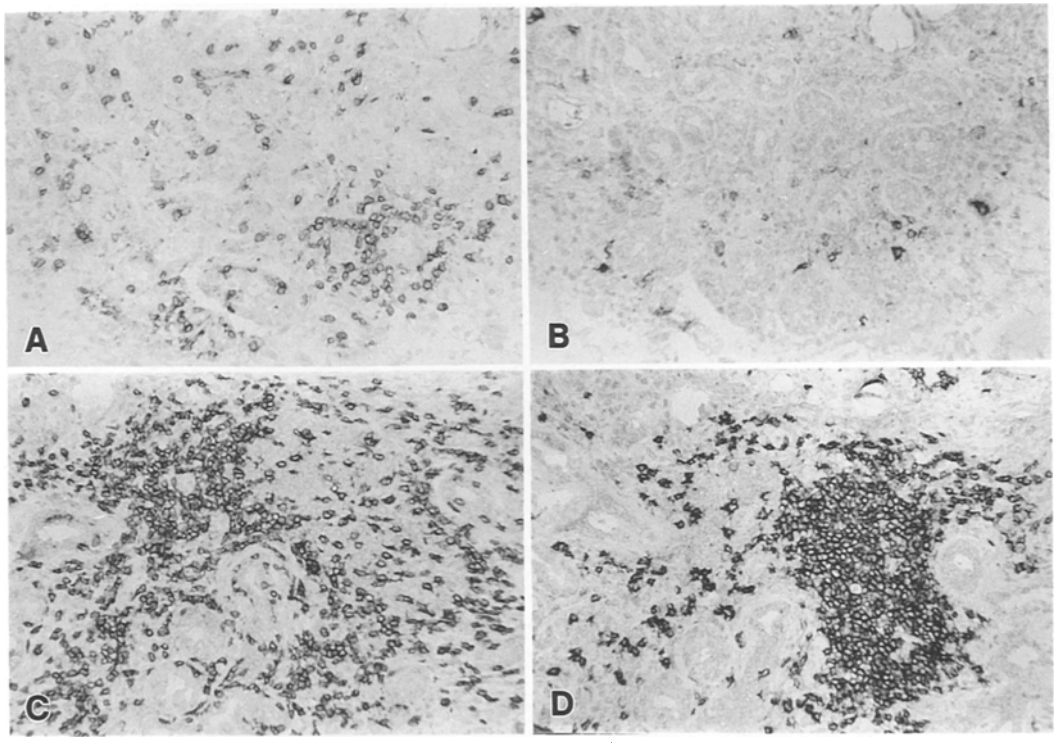

Fig. 2. Areas of diffuse (**A, B**) and focal (**C, D**) lymphocytic infiltrate within serial sections of a labial gland from a patient with primary Sjogren's syndrome stained for CD3 (Leu4; **A, C**) and CD20 (L26; **B, D**) lymphocytes. Immunoperoxidase-haematoxylin, ×100

had significantly higher T:B-cell ratios (7.3±4.4:1) compared to focal infiltrates (2.4±1.9:1; $P<0.001$). B cells were normally present within the central areas of large foci and surrounded by T cells (Fig. 2). The ratio of $CD8^-$ to $CD8^+$ T cells was relatively constant throughout the specimens and no significant differences were detected between focal (2.6±1.2:1) and diffuse (2.8±1.1:1) areas of infiltration. Whilst primed $CD8^-$ T cells predominated over the naive subset within all glandular infiltrates the ratio of the primed to naive phenotype was significantly higher in lymphoid foci (4.2±1.5:1) compared to diffusely infiltrated areas (1.5±0.8:1; $P<0.001$). The percent contribution of each lymphocyte subset in focal and diffuse areas of infiltration, determined from the cell ratios, are shown in Table 3. Compared to diffuse infiltrates, foci contain proportionally more B cells and less T cells, $CD8^-$ cells and naive $CD8^-$ cells than diffuse infiltrates. Primed $CD8^-$ cells appear to account for about 40% of lymphocytes irrespective of type of infiltrate.

Table 3. Percentage contribution of lymphocyte types and subsets to focal and diffuse infiltrates

Lymphocyte	Type of infiltrate	
	Focal	Diffuse
T + B cells	100	100
B cells	27[a]	12
T cells		
$CD3^+$	73 (100)	88 (100)
$CD8^+$	20.3 (27.8)	23.2 (26.4)
$CD8^-$	52.7 (72.2)	64.8 (73.6)
Primed $CD8^-$	42.6 (58.4)	38.9 (44.2)
Naive $CD8^-$	10.1 (13.8)	25.9 (29.4)

[a] Percentage of lymphocytes derived using mean ratios shown in Table 2 for areas of focal and diffuse infiltration. Values in parentheses are T subsets as percentage of T cells

The overall preponderance of T cells of the primed phenotype was confirmed by the large numbers of lymphocytes within known T-cell areas which stained for CD48RO using the monoclonal antibody UCHL1 (Fig. 3) and the similar memory:naive ratios observed in three specimens which were also stained using antibody combinations including both UCHL1 and WR16 (Table 4).

Discussion

Using a sequential double immunoperoxidase method we have demonstrated significant differences in the distribution of T and B cells and primed and naive helper T cells within focal and diffuse inflammatory infiltrates in labial salivary glands from patients with Sjogren's syndrome. Foci contained more B cells and exhibited

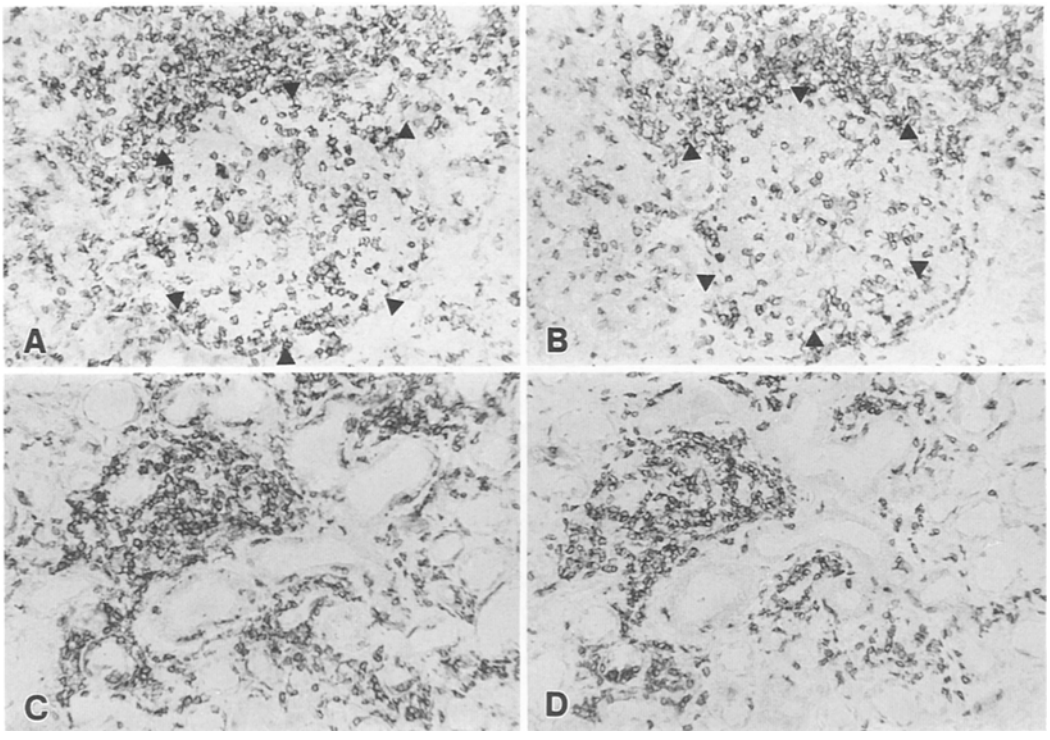

Fig. 3. Serial sections of labial glands containing large (**A, B**) and medium-sized (**C, D**) infiltrates stained for CD3 (Leu4; **A, C**) and CD45RO (UCHL1; **B, D**). Many of the negative cells within the *arrowed* area are B lymphocytes. Immunoperoxidase-haematoxylin, ×100

Table 4. Primed:naive ratios in three specimens determined using different monoclonal antibody combinations

Specimen	Primed:naive cell ratios[a]							
	Focal infiltrates				Diffuse infiltrates			
	(3:5)	(3:4)	(6:4)	(6:5)	(3:5)	(3:4)	(6:4)	(6:5)
1	2.5:1	2.3:1	2.0:1	2.2:1	1.5:1	1.2:1	1.0:1	1.3:1
2	3.9:1	3.5:1	2.8:1	3.1:1	1.3:1	1.6:1	0.9:1	0.7:1
3	5.4:1	4.2:1	6.0:1	7.7:1	ND	ND	ND	ND
Mean	3.9:1	3.3:1	3.6:1	4.3:1	1.4:1	1.4:1	1.0:1	1.0:1

ND, Diffuse infiltrates not present

[a] Ratio determined using antibody combinations 3 and 5, 3 and 4, 6 and 4 and 5 and 6 (Table 1)

significantly higher primed:naive helper T-cell ratios than diffuse infiltrates. By contrast, the ratio of $CD8^+$ to $CD8^-$ T cells was similar in all lymphoid infiltrates. This distribution was the same in glands from patients with both primary and secondary Sjogren's syndrome.

The predominance of primed helper T cells over the naive subset within focal infiltrates (about 81% of $CD8^-$ T cells) of salivary glands from patients with Sjogren's syndrome agrees with previous double (Modlin et al. 1988; Moore et al. 1988; Markey et al. 1990) and single (Moore et al. 1988; Stein-Oakley 1989; Katayama et al. 1990) immunocytochemical studies at other inflammatory sites. The low primed:naive helper T-cell ratios in diffuse glandular infiltrates have not been previously reported in inflammatory lesions other than those associated with lepromatous leprosy (Modlin et al. 1988) where it has been interpreted as reflecting the known hyporesponsive state to *Mycobacterium leprae* in patients with this condition. Although peripheral blood lymphocytes were not investigated in this study, it is interesting to note that the T:B and primed:naive helper T-cell ratios in diffuse infiltrates are similar to those reported in the circulation of patients with Sjogren's syndrome (Sato et al. 1987). However, simultaneous analysis of peripheral blood and lesional tissues has demonstrated selective accumulation of the primed helper T cells within rheumatoid synovium (Moore et al. 1988) and lesions of tuberculoid leprosy (Modlin et al. 1988). The finding of significantly higher primed to naive helper T-cell ratios in focal compared with diffuse salivary gland infiltrates suggests that a similar selective accumulation of the primed subset may also occur locally in Sjogren's syndrome.

If many of the small collections of lymphocytes represent sites of future focal infiltration, our data suggest that an initial, almost exclusively, T-cell-rich glandular infiltrate, containing primed and naive helper populations in similar proportions to that found in peripheral blood, progresses to lymphoid foci containing significant numbers of B cells and a selectively increased primed helper T-cell population. This increased primed-to-naive ratio might reflect selective migration and/or retention within the site, in situ proliferation or phenotypic conversion after entering the site due to mediators in the microenvironment. That selective migration and/or retention might occur is supported by the known high level of expression by primed cells of surface adhesion molecules capable of binding to many cell types including endothelial cells (LFA-1, CD2) and, possibly, fibronectin (CD29). Indeed, in vitro experiments have demonstrated that primed cells are more effective than naive cells in binding to endothelial cells under various culture conditions (Pitzalis et al. 1988). By contrast, in situ proliferation seems less likely as there are relatively low numbers of activated, interleukin-2 receptor expressing T-cells, antigen-specific cells (Modlin et al. 1988) or Ki67-positive proliferating cells (Matthews and Mason, unpublished observations) demonstrable within lesions. Although phenotypic conversion to the primed phenotype has been demonstrated in vitro in response to various stimuli (Akbar et al. 1988; Salmon et al. 1989), evidence for in vivo conversion at inflammatory sites is lacking. Whilst our data do not differentiate between these possibilities, if the diffuse infiltrates do represent the initial glandular lesion and if selective migration is important in accumulation of primed cells, then local factors, released after initial infiltration of the site, must be involved.

There is no direct evidence that the functions ascribed to the primed and naive helper T subsets from in vitro experiments occur in vivo. However, the close anatomical assocation between T cells of the primed phenotype and B-cell foci, thought to contain expanded clones of $CD5^+$ (Youinou et al. 1988) and rheumatoid factor cross-reactive idiotype expressing B cells (Fox et al. 1986a; Deacon et al. 1991) also suggests a functional relationship between these cell types. It is interesting to speculate that a local increase in helper-inducer function might be one factor responsible for upregulated B-cell proliferation which is reflected in the known increased risk of lymphoproliferative disorders in Sjogren's syndrome including malignant lymphoma of the salivary glands (Talal and Bunim 1964; Kassan et al. 1978; Schmid et al. 1989).

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