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ORIGINAL ARTICLE

Immunoprofile of focal lymphocytic infiltration in minor salivary glands of healthy individuals

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AIM: To characterize the immunohistochemical profile of the inflammatory cells included in the focal lymphocytic infiltration in the minor salivary glands of healthy individuals.

MATERIALS AND METHODS: Tissue samples of the labial and palatal salivary glands from 46 postmortem subjects, demonstrating the presence of focal lymphocytic infiltration were quantitatively evaluated for the presence of T- and B-cell lymphocytes, plasma cells and macrophages by immunohistochemical and morphometric methods.

RESULTS: B-cell lymphocytes, the predominant cell population in labial (67.5%) and palatal salivary glands (60.8%), were more frequent than T-cell lymphocytes in both glands ($P < 0.001$). Among the T-cell lymphocytes, CD_4-positive cells were significantly more prevalent than the CD_8-positive cells ($P < 0.001$). Plasma cells were almost absent, comprising only 0.01% of the focal lymphocytic infiltration cells of the labial and palatal salivary glands.

CONCLUSIONS: Focal lymphocytic infiltration in the samples of the salivary glands obtained from healthy individuals is devoid of plasma cells. This can serve as an additional means to differentiate between focal lymphocytic infiltration in patients with Sjögren's syndrome, in which plasma cells are abundant, and focal lymphocytic infiltration in individuals with other causes of focal sialadenitis.

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Introduction

Focal lymphocytic infiltration (FLI) is the histopathological hallmark of Sjögren's syndrome (SS) (Fox and Speight, 1996). Occasionally, FLI is found in the major salivary glands (Kurashima and Hirokawa, 1986) and the minor salivary glands (Scott, 1980; Syrjanen, 1984; De Wilde *et al*, 1986; Takeda and Komori, 1986; Vered *et al*, 2001; Radfar *et al*, 2002) of healthy individuals. Focal sialadenitis in healthy individuals might represent an immunological response to a topical, exogeneous stimulus that penetrates into the gland parenchyma by means of a retrograde passage through the ductal system of the salivary glands (Vered *et al*, 2001). The cellular components of these foci have been thoroughly investigated in tissue samples from SS (Chused *et al*, 1974; Konttinen, 1981; Dalavanga *et al*, 1986; Segerberg-Konttinen *et al*, 1987; Prochorec-Sobieszek *et al*, 2004). However, there are no data in the literature regarding the cellular characterization in FLI of healthy individuals.

The purpose of the present study was to characterize the immunohistochemical profile of the different inflammatory cells composing FLI in the minor salivary glands of healthy individuals, with histomorphometric methods.

Materials and methods

Tissue specimens

The study group consisted of 46 post-mortem tissue samples of the labial salivary glands (LSG, $n = 10$) and palatal salivary glands (PSG, $n = 36$) of healthy individuals, which demonstrated the presence of FLI. Samples were selected from a large series of LSG and PSG as previously reported (Vered *et al*, 2001), and included only those classified as FLI of grade 2 and grade 3 according to the modified Chisholm and Mason grading system (Chisholm and Mason, 1968) (Table 1). In five cases, FLI within PSG and LSG of the same subject were identified. Subjects were unaffected by

Table 1 Modified Chisholm and Mason grading for FLI[a]

Grade	*No. lymphocytic foci*
1	No. lymphocytic foci
2	FLI of less than one focus
3	FLI of one focus or more than one focus

[a]Measurements taken on 4 mm^2 of salivary gland tissue.

autoimmune diseases, leukemia, malignant lymphoma, tumors of the head and neck, or local infectious diseases, and did not receive any cytotoxic drugs prior to death (Scott, 1980). Death was caused by road accidents, gunshot wounds, cardiac arrest, traumatic injuries, drowning, or suicide. Postmortem examination and tissue harvesting were performed within approximately 6 h after death. Fixation of the salivary gland tissue samples was performed immediately.

Immunohistochemical stains

All samples were cut into 3 μm width mounted on positive-charged microscope slides (Optiplus^{TM}; Biogenex, San Ramon, CA, USA). After dewaxing in xylene, sections were dehydrated in ethanol, placed in 3% H_2O_2 for 10 min, and rinsed in distilled water for 10 min. The avidin–biotin complex (ABC) method was used to demonstrate immunohistochemical staining, according to the manufacturer's instructions. Commercially available primary antibodies were used to demonstrate the presence of different inflammatory cells within FLI: lymphocytes of T and B type, T lymphocytes of CD_4 and CD_8 subtypes, macrophages and plasma cells. Table 2 lists the antibodies used, the source and the dilution. Tissue samples of reactive lymph nodes served as positive control for the different antibodies and exclusion of the primary antibodies served as negative controls.

Histomorphometric evaluation

From each sample, sections were cut serially to provide the complete FLI immunoprofile. Sections were evaluated with a light microscope (Olympus BH2, Tokyo, Japan) on which an ocular with an engraved 100-square grid (Olympus BH2) was assembled. Initially, the total number of inflammatory cells (immunohistochemically stained and non-stained cells) (ΣInfCell) for all FLI present in each section, was assessed by examining each slide at magnification ×40. After identifying one FLI, the grid was positioned over the FLI at ×400 magnification. The number of inflammatory cells that occupied all the 10 squares located along the diagonal of the grid, starting from its uppermost left square to the lowest right square, was counted. Thus, the mean number of inflammatory cells per square for that slide was calculated. The total number of squares occupied by all FLI in each section was multiplied by the mean number of inflammatory cells per square. The same method was used to assess the total number of positively immunostained inflammatory cells. This was performed for each antibody, where ΣInfPC represents the total number of positively stained plasma cells, ΣInfBL and ΣInfTL the total number of B and T cell lymphocytes, respectively, $\Sigma InfCD_4$ and $\Sigma InfCD_8$ for CD_4 and CD_8 positively stained T lymphocytes, respectively, and ΣInfCD68 for the total number of macrophages. The total number of these cells was calculated by multiplying the mean number of stained cells in each square by the number of squares occupied by the stained cells for each antibody. The percentage of the positively stained cells from all inflammatory cells in each section for each antibody was calculated by dividing ΣInfPC, ΣInfBL, ΣInfTL, etc. by ΣInfCell and multiplying the result by 100. When only a few immunohistochemically stained cells (for any of the examined antibodies) were found, all the cells were counted precisely but not averaged, and their percentage calculated. The results are presented as the mean percentage of the positively stained cells for each antibody in LSG and PSG.

Statistical analysis

Differences between the percentage of B and T cells, and CD_4 and CD_8 cells at each location (LSG and PSG) were assessed by the Wilcoxon signed-rank test. Differences between percentages of the different types of inflammatory cells among the five cases, in which both LSG and PSG demonstrated FLI, were evaluated by the Wilcoxon signed-rank test. Correlations between percentage of the different types of inflammatory cells, histologic grading, and location were analyzed using the Mann–Whitney test. Computations were made using the Statistical Package for the Social Sciences (SPSS, 13.0) software. All results were statistically significant at $P < 0.05$.

Results

The study included samples from 19 males and 17 females (age range 17–91 years, mean 59.1 years) in the PSG group and three males and seven females (age range 20–82 years, mean 57.4 years) in the LSG group. The samples fulfilled the criteria of FLI grade 2 and 3 as previously mentioned. Table 3 summarizes the

Table 2 Antibodies used to determine the immunoprofile of the different inflammatory cell populations within the FLI

Type of cell	*Antibody*	*Source*	*Clone*	*Dilution*
B lymphocytes	Pan B (CD20)	Dako A/S (Glostrup, Denmark)	L26	1:400
T lymphocytes	Pan T (CD45Ro)	Dako A/S (Glostrup, Denmark)	UCHL1	1:400
T lymphocytes	CD_4	Zymed (San Francisco, CA, USA)	ZT-17	1:50
T lymphocytes	CD_8	Zymed (San Francisco, CA, USA)	1A5	1:50
Plasma cells	CD138	Serotec (Kidlington, UK)	B-B4	1:100
Macrophages	CD68	Dako A/S (Glostrup, Denmark)	PG-M1	1:100

Table 3 Distribution of examined gland samples according to age and histologic grading of the FLI

	PSG		*LSG*	
Age	*Grade 2*	*Grade 3*	*Grade 2*	*Grade 3*
< 30 years	3	0	1	0
31–60 years	10	2	1	2
> 60 years	18	3	5	1
Total	31	5	7	3

distribution of the examined samples according to age and FLI grades. Most samples ($n = 27$, 58.7%) were from individuals over 60 years of age. The percentages of the B and T cells in each examined case in LSG and PSG are shown in Figure 1a, and the percentages of the CD_4 and CD_8 cells in Figure 1b.

B cells were the predominant cell population in both LSG and PSG, averaging 67.5% (±10.7) of the FLI in LSG and 60.8% (±20.6) of the FLI in PSG (Figure 2). The frequency of T cells was about three times less than that of B cells in both LSG (23.5% ± 7.3) and PSG (20.3% ± 13.3) (Figure 3). The difference between the percentage of B and T cells in both LSG and PSG was statistically significant ($P < 0.001$).

Among the T-cell population, CD_4-positive cells (Figure 4) were remarkably more prevalent than the CD_8-positive cells (Figure 5). Of the FLI cells, CD_4- and CD_8-positive cells consisted of 32.1% (±13.3) and 0.9%

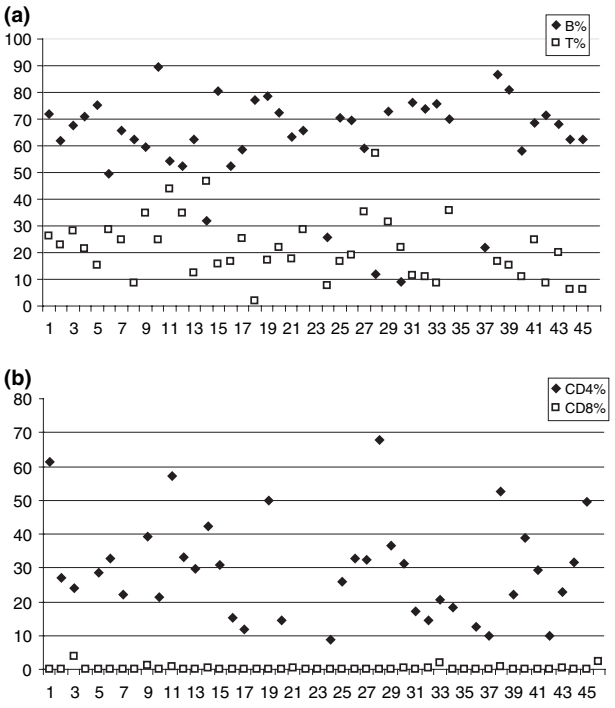

Figure 1 The percentage of B and T cells (**a**) and CD_4 and CD_8 cells (**b**) in each examined case. Cases 1–10 are from the labial salivary glands (LSG) and cases 11–46 are from the palatal salivary glands (PSG)

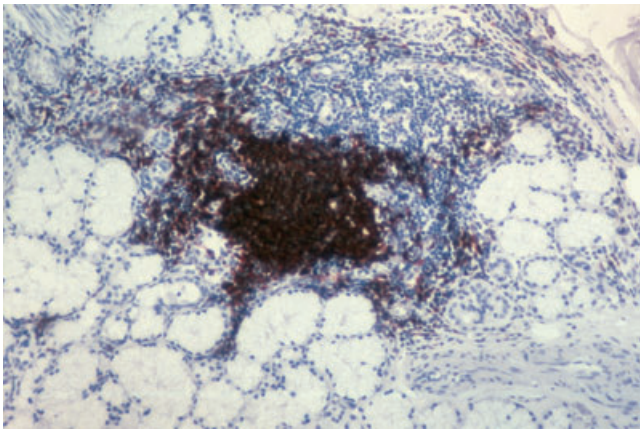

Figure 2 Photomicrograph shows a section of palatal salivary glands immunostained for B cells (CD20). The large focus of lymphocytes is located adjacent to conserved acinar structures (lower left corner). The majority of the lymphocytes within this focus demonstrate intense immunoreactivity for CD20 (ABC method, original magnification ×100)

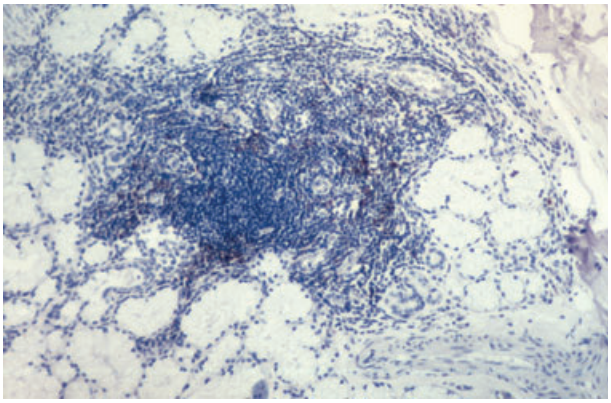

Figure 3 Photomicrograph showing the same section as in Figure 2, immunostained for T cells (CD45Ro). Only a minority of the lymphocytes within this focus yielded a positive immunoreaction for CD45Ro (ABC method, original magnification ×100)

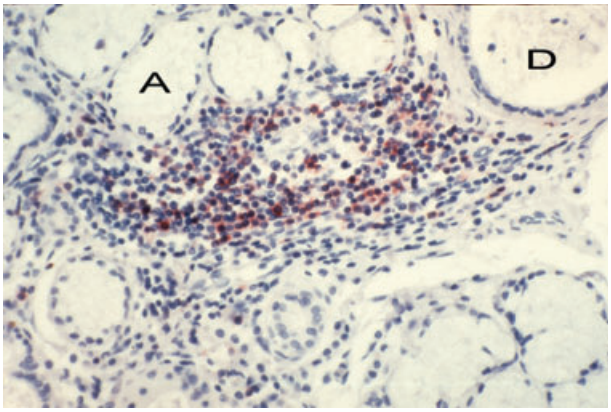

Figure 4 Photomicrograph shows a small focus of lymphocytes in palatal salivary glands immunostained for T lymphocytes of the CD_4 subtype. Normal acinar (A) and ductal (D) structures are clearly observed. Positive immunoreactivity was found in most of the lymphocytes within this focus (ABC method, original magnification ×200)

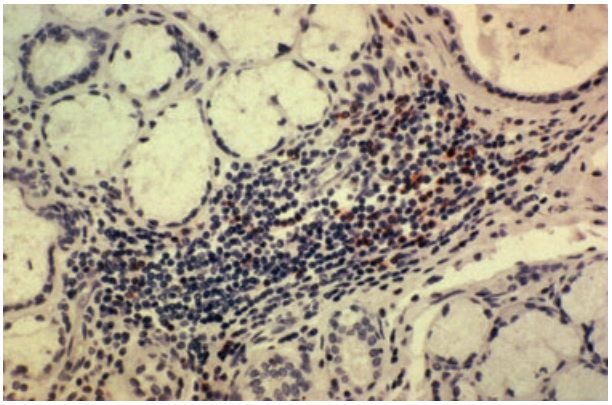

Figure 5 Photomicrograph of the same section as in Figure 4, immunostained for T lymphocytes of the CD_8 subtype. Only a few scattered cells exhibited positive immunoreactivity for CD_8 (ABC method, original magnification ×200)

(±1.6) of the LSG, respectively, and 30.1% (±16.3) and 0.4% (±0.7) of the PSG, respectively. The difference between the percentage of CD_4- and CD_8-positive cells in both LSG and PSG was statistically significant ($P < 0.001$).

Only isolated macrophages were found within FLI in both LSG and PSG, which consisted of approximately 0.04% of all cells. Similarly, plasma cells were also very scarce, approximately 0.01% of all the cells in FLI in both LSG and PSG.

No correlation was found in the percentage of the different types of inflammatory cells within FLI between LSG and PSG ($P > 0.1$). Furthermore, the histologic grading of the examined FLI was not correlated significantly with the percentage of the different types of inflammatory cells in the sections examined.

Discussion

The present study further investigated the immunohistochemical properties of FLI in LSG and PSG of healthy individuals reported in a previous study (Vered *et al*, 2001). To the best of our knowledge, this is the first study to present the characterization of inflammatory infiltrate components, i.e. lymphocytes and plasma cells in FLI. The predominant inflammatory cell type in FLI of both LSG and PSG in healthy individuals was the B cells, which consisted of approximately two-thirds of the cells within the examined FLI. In contrast, approximately one-fourth of the cells within the same assessed FLI were T cells. CD_4-positive cells were more prevalent than CD_8 among the T cells. An insignificant number of macrophages and plasma cells was scattered throughout the FLI. The total percentage of inflammatory cells in LSG and PSG did not add up to 100%. Given the negligible numbers of macrophages and plasma cells, it seemed likely that about 10–20% of the inflammatory cells were null lymphocytes. It can be assumed that a small part of the antigenic epitopes was destroyed as a result of postmortem changes in the tissue; however, it is probable that these changes affected the antigenic epitopes on all types of cells equally. Therefore, it was expected that even if all the antigenic epitopes would have been targeted by the immunostain, the relative proportions of the various types of cells would basically remain the same.

The presence of FLI in minor salivary glands is usually the hallmark of the histopathologic features of several immunologically mediated conditions especially SS. Semiquantitative assessment of T- and B-cell lymphocytes in SS revealed contradictory results as to the predominant cell components of FLI – T cells (Dalavanga *et al*, 1986; Segerberg-Konttinen *et al*, 1987) or B cells (Chused *et al*, 1974; Konttinen, 1981; Prochorec-Sobieszek *et al*, 2004). The relative frequency of T-cell sub-populations in the FLI of SS patients showed a considerably higher percentage of CD_4 lymphocytes compared with CD_8 (Fox and Speight, 1996; Xanthou *et al*, 1999; Prochorec-Sobieszek *et al*, 2004). The present study of healthy individuals showed FLI in both LSG and PSG, which consisted mostly of B lymphocytes. The T-lymphocyte population showed that the CD_4 subtype was much more prevalent than the CD_8 subtype, which is in accordance with the findings in SS patients.

In SS patients, plasma cells are consistently found in the FLI of LSG and are postulated to play a pivotal role in the pathogenesis of this disease. In the LSG of SS patients, an increased secretion of immunoglobulins has been found compared with glands from patients with rheumatic arthritis or systemic lupus erythematosus, without evidence of SS (Talal *et al*, 1970). These findings are supported by Anderson *et al* (1972), who further demonstrate an increased number of IgM- and IgG-positive plasma cells compared with the salivary glands from patients with various connective tissue diseases. The total amount of plasma cells, especially those positive for IgM and IgG, was significantly higher in SS patients compared with patients with glands showing non-specific inflammatory changes and with normal LSG (Lane *et al*, 1983; Speight *et al*, 1990). These findings could be used as a diagnostic tool of SS (Bodeutsch *et al*, 1992; Fox and Speight, 1996). In the present study, the absence of plasma cells in the FLI of the healthy population emphasized the difference between the FLI found in SS patients and that found in healthy individuals.

In summary, LSG biopsy adds little useful information in patients where the diagnosis of SS is clinically obvious. It has been suggested that lip biopsies be mandatory in patients who fulfill only partial criteria for SS and positive anti-Ro or anti-La antibodies (Lee *et al*, 1998). The results of the present study suggest that immunohistochemical staining for plasma cells of LSG samples can differentiate between FLI of SS and FLI from individuals with other causes of focal sialadenitis. FLI from SS patients characteristically contain an abundance of plasma cells, in contrast to FLI from non-SS patients, in which plasma cells are almost absent.

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