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### Authors

Fox, RI  
Hugli, TE  
Lanier, LL  
et al.

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## SALIVARY GLAND LYMPHOCYTES IN PRIMARY SJOGREN'S SYNDROME LACK LYMPHOCYTE SUBSETS DEFINED BY LEU-7 AND LEU-11 ANTIGENS<sup>1</sup>

ROBERT I. FOX,<sup>2\*</sup> TONY E. HUGLI,<sup>†</sup> LEWIS L. LANIER,<sup>§</sup> EDWARD L. MORGAN,<sup>3\*</sup> AND FRANK HOWELL<sup>†</sup>

From the Departments of \*Basic and Clinical Research, †Immunology, and ‡Oral Medicine, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037; and the §Becton Dickinson Monoclonal Center, 2375 Garcia Avenue, Mountain View, CA 94043

Primary Sjogren's Syndrome (SS) is an autoimmune disease characterized by dry eyes and dry mouth due to lymphocytic infiltration of lacrimal and salivary glands. Biopsies of their salivary glands provided an opportunity to characterize the phenotypic and functional properties of inflammatory site lymphocytes. We found that the salivary gland lymphocytes (SGL) of SS patients differed from the peripheral blood lymphocytes of the same patients because: a) SGL lacked lymphocytes reactive with anti-Leu-7 and anti-Leu-11 monoclonal antibodies; b) SGL lacked natural killer (NK) activity; and c) SGL lacked the ability to suppress polyclonal B cell responses in the presence of complement fragment C3a, a function that requires the presence of Leu-7<sup>+</sup> cells. These studies also showed that the SGL of SS patients differed from tonsillar lymph node (LN) lymphocytes of immunologically normal individuals because tonsillar LN contained Leu-7<sup>+</sup> T cells, and tonsillar LN could suppress polyclonal B cell responses in the presence of the complement fragment C3a. The absence of this regulatory subset in the salivary glands of SS patients may contribute to pathogenesis, because these cells may be important in the suppression of polyclonal antibody synthesis and in the elimination of neoplastic or viral infected cells.

Sjogren's Syndrome (SS)<sup>4</sup> is an autoimmune disorder of unknown etiology. Affected patients typically have dry eyes (keratoconjunctivitis sicca, KCS), dry mouth (xerostomia), hyperglobulinemia, and elevated titers of autoantibodies (including rheumatoid factor and anti-nuclear antibodies) (1-3). Their salivary glands, which are infiltrated by lymphocytes, become active sites of autoantibody production (4). Patients are at increased risk for

non-Hodgkin's lymphoma (5), so that biopsy of persistently enlarged salivary glands may be required for diagnostic purposes. In patients lacking lymphoma, these biopsies provide a source of inflammatory site lymphocytes for phenotypic and functional analysis (6). Previous studies have shown that the majority of salivary gland lymphocytes (SGL) were T cells (Leu-4<sup>+</sup>) that included both Leu-3<sup>+</sup> (T helper) and Leu-2<sup>+</sup> (T cytotoxic) subsets (6-8). The SGL of SS patients differed from the peripheral blood lymphocytes (PBL) of the same individuals in several regards: a) a higher proportion of Leu-3<sup>+</sup>/Leu-2<sup>+</sup> cells was present in SGL than PBL in most patients; b) a higher proportion of "activated" T cells (i.e., T cells bearing OKT10 and HLA-DR antigens) was found in the salivary gland; and c) a novel B cell subset (defined by monoclonal antibody B532) was present in their SGL but not in their blood (6-8). The present study extends these observations by demonstrating the absence of Leu-7<sup>+</sup> or Leu-11<sup>+</sup> subsets in the SGL of SS patients despite the presence of these cells in their blood.

We also wished to compare the SGL of SS patients to the lymphocytic tissue infiltrates present in immunologically normal individuals. Because normal salivary glands generally lack lymphocytic infiltrates (1, 3), we have studied tonsillar lymph nodes (LN) from patients lacking autoimmune or neoplastic disease. These LN represent sites of chronic inflammation in the oropharynx (9), a site in proximity to the salivary glands. Tonsillar LN were similar to SGL in their predominance of T cells of the Leu-3<sup>+</sup> subset and in their content of B532<sup>+</sup> B cells (10-12). However, tonsillar LN contained Leu-7<sup>+</sup> cells (13) that we now report as absent in SGL. To extend our comparison of tonsillar LN and SGL, we also have used *in vitro* functional assays. We have emphasized the polyclonal stimulation of B cell activity by Fc fragments of human IgG, because recent studies in our laboratories have suggested that this stimulation can be suppressed by the complement fragment C3a (14), and that this "suppression" was dependent on Leu-7<sup>+</sup> cells (unpublished observations). Our results indicate that SGL are deficient in this suppressor function and thus may lack an important immunoregulatory circuit at the site of inflammation.

### MATERIALS AND METHODS

*Patient population and source of lymphoid tissues.* Primary SS was defined by the presence of KCS, xerostomia, and a class IV lip biopsy (on the Chisholm-Mason scale from I to IV) (1, 7). Major salivary gland (submandibular and/or parotid) biopsies were obtained from six patients with SS (aged 19 to 74 yr). These biopsies

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<sup>2</sup> Direct reprint requests to: Robert I. Fox, M.D.

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<sup>4</sup> Abbreviations used in this paper: SS, Sjogren's Syndrome; SGL, salivary gland lymphocyte; LNL, lymph node lymphocyte; 2MGP, the carboxypeptidase inhibitor 2-mercaptomethyl-S-guanidino pentanoic acid.

showed characteristic changes of SS without evidence of lymphoma or infection (6, 8). Minor salivary gland biopsies from the lower lip of 15 SS patients were used for analysis of lymphocyte subsets by immunohistologic analysis of frozen tissue sections (7). These patients did not meet the criteria for rheumatoid arthritis, systemic lupus erythematosus, or scleroderma, although many exhibited a number of extraglandular features including thyroid disease, interstitial nephritis, interstitial pneumonitis, or peripheral neuropathy (1). None had received corticosteroids or immunosuppressive medications for at least 3 mo before biopsy.

Salivary gland tissue from four otherwise normal patients with salivary gland adenomas containing extensive lymphocytic infiltrates were obtained at the time of surgery (9). These patients lacked serum autoantibodies or hyperglobulinemia, and had no evidence of a connective tissue disease. Normal salivary gland tissue was obtained from the normal margins of certain salivary gland tumors (three cases), from fresh autopsy specimens (two cases), or from minor salivary gland biopsies performed to rule out SS (grade 1 on the Chisholm-Mason scale) (1, 7) in eight cases.

Tonsillar LN were removed from six immunologically normal adults (aged 18 to 62 yr). Three LN were obtained at the time of abdominal surgery from immunologically normal individuals (aged 35 to 65 yr). These LN were normal based on routine histologic analysis and immunologic characterization of cell surface markers (described below). Because the *in vitro* functional results and proportions of lymphocyte subsets were similar for tonsillar and LN lymphocytes, we have grouped the results of these lymphocytes as normal LN lymphocytes (LNL).

**Monoclonal antibodies.** Antibody SC1, produced in our laboratory, detects a 67,000 dalton antigen on T cells that is similar to that reactive with antibody Leu-1 (15). The properties of monoclonal antibodies against Leu-2 (suppressor/cytotoxic subset), Leu-3 (helper/inducer subset), Leu-4 (mature T cells), and Leu-5 (pan T cell) (Becton Dickinson, Mountain View, CA) antigens have been described (16). These antibodies recognize subsets equivalent to those defined by antibodies OKT8, OKT4, OKT3, and OKT11, respectively (17). Although the phenotype of a particular subset may suggest its function, the correlation between phenotype and function is often imprecise (18, 19). Mononuclear cells with natural killer (NK) activity react with anti-Leu-11 (Becton Dickinson) (20). Antibody F45, produced by Fraenkel (21) and additionally characterized in our laboratory (22), recognizes the same 110,000 dalton glycoprotein as antibody anti-Leu-7. However, antibodies F45 and anti-Leu-7 detect distinct epitopes based on competition studies (unpublished observations). Similar results were obtained with each antibody in flow cytometric and immunohistologic studies (22). Antibodies anti-Leu-7 and F45 are not specific for hematopoietic cells, because cells of neuroectodermal origin including myelinated nerve, argentaffin cells in colon, acinar cells in prostate or salivary gland, and islet cells in the pancreas, exhibit reactions with this antibody (23). However, these cells can be distinguished from lymphoid cells based on their morphologic appearance. Antibody OKM1 (Ortho Diagnostics, Raritan, NJ) detects the complement C3bi receptor (24); cells bearing OKM1 antigen include monocytes, granulocytes, NK, cells and at least one subset of T suppressor cells (25, 26).

B cells were recognized by their reactivity with anti- $\kappa$ , anti- $\lambda$ , anti-IgD-anti-Leu-12 (Becton Dickinson), and OKB7 (Ortho). A subpopulation of B cells in the germinal centers of normal LN and primary SS salivary glands was reactive with antibody B532 (produced by Dr. D. Frisman of the University of California at San Diego); PBL of normals or primary SS patients were unreactive with this antibody (7). Control myeloma proteins of the IgG1, IgG2, and IgM classes were used at the same concentrations as specific monoclonal antibodies.

**Preparation of lymphocyte suspensions and staining with monoclonal antibodies.** Lymphocytes were obtained from salivary gland (SGL) or from lymph node (LNL) by gently passing the minced tissue through a stainless steel mesh. These LNL or PBL were centrifuged over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Biopsies of major salivary glands or LN generally yielded  $5 \times 10^7$  to  $5 \times 10^9$  lymphocytes. For flow cytometric analysis, aliquots were stained by monoclonal antibodies and fluorescein-conjugated F(ab')<sub>2</sub> goat anti-mouse Ig (Tago, Burlingame, CA) (6, 27). Statistical comparisons of the proportion of cells stained with particular monoclonal antibodies were made by using the Student's *t*-test. For two-color immunofluorescent studies, fluorescein-conjugated antibodies (anti-Leu-7 or anti-Leu-11a) and phycoerythrin-conjugated antibodies (anti-Leu-2, anti-Leu-3, anti-Leu-4, or anti-HLA-DR) were used. Fluorescein and phycoerythrin were excited by using a single argon ion laser (488 nm, 200 mW). Low angle forward light scatter, right angle light scatter, and green fluorescence and red fluorescence signals were determined by using a FACS IV system, and data were stored in list mode data files by using a Consort 40 PDP III-based computer

(Becton Dickinson FACS Division, Sunnyvale, CA). Two-parameter data were collected into a 64 by 64 matrix and were displayed as contour maps. Contours were drawn to indicate increasing numbers of cells in a defined area of the array. Areas within the contour map were integrated to determine the percentage of cells (20). In addition to flow cytometric analysis, the stained cells were cytocentrifuged onto slides, and were read in a blinded manner by using a Zeiss fluorescent microscope with epi-illumination and appropriate barrier filters. As controls, myeloma proteins with no anti-human activity (including fluorescein- and phycoerythrin-conjugated antibodies for two-color stains) were used to detect nonspecific binding.

**Immunoperoxidase staining of frozen tissue sections.** Fresh surgical biopsy specimens were embedded in OCT compound (Tissue Tek Inc.) and were frozen at  $-70^\circ\text{C}$  until  $5 \mu\text{m}$  sections were prepared, were fixed in acetone, and were stained as described (7). Briefly, the slides were treated with specific monoclonal antibody or control myeloma protein. After rinsing, the sections were treated with biotin-conjugated F(ab')<sub>2</sub> anti-mouse Ig (Tago), avidin-conjugated horseradish peroxidase (Vector, Burlingame, CA), and substrate 3',3'-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO). Slides were counterstained with methylene blue and were read in a blinded manner.

**Preparation of complement components and Ig Fc fragments.** Human C3a was isolated from complement-activated serum containing the carboxypeptidase inhibitor 2-mercaptomethyl-5-guanidino pentanoic acid (2MGP) at 1 mM, as described (28, 29). C3a desArg was prepared by the same procedure in the absence of carboxypeptidase inhibitor. The C3a and C3a desArg were judged to be >95% homogeneous by N-terminal peptide analysis, the presence of a single-band pattern on cellulose acetate strip electrophoresis, and amino acid composition. Human C3a preparations contained <0.1% C4a or C5a, as determined by radioimmunoassay (28). Fc fragments from a human IgG1 myeloma protein (a generous gift from Dr. H. Spiegelberg) were obtained by papain digestion (Sigma) in the presence of L-cysteine and EDTA for 18 hr, followed by DEAE chromatography (30).

**Assay of NK activity.** Lymphocyte suspensions of PBL, LNL, or SGL were incubated at  $37^\circ\text{C}$  for 4 hr with  $^{51}\text{Cr}$ -labeled K562 cells (21). The lymphocytes and target cells were mixed at effector target ratios of 20:1 and 60:1, as described (22).

**Polyclonal antibody responses.** Suspension of PBL, LNL, or SGL ( $3 \times 10^5$  cells) in 0.3 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1% BME vitamins, 100  $\mu\text{g}$  penicillin, 100  $\mu\text{g}$  streptomycin, 1  $\mu\text{g}/\text{ml}$  Fc fragments, and 10% fetal calf serum (GIBCO, Grand Island, NY) were placed in flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA) for 6 days at  $37^\circ\text{C}$  in a humidified, 10% CO<sub>2</sub> atmosphere. Cell cultures were done in quadruplicate. The number of Ig-secreting cells was determined by the protein A plaque assay (30). Briefly, lymphoid cell suspensions were mixed with protein A-coupled sheep erythrocytes in agar. After 1/2 hr at  $37^\circ\text{C}$ , amplifying sera recognizing human IgG + IgM + IgA and guinea pig complement were added. The results are recorded as Ig-secreting cells/ $10^6$  original mononuclear cells  $\pm$  SE. Previous studies have demonstrated that optimal polyclonal responses after Fc fragment stimulation are detected on day 6 (6, 30). The stimulation by Fc fragments is not dependent on the particular IgG1 myeloma protein utilized in these studies, because Fc fragments from pooled IgG also are effective (30). The use of a myeloma protein has the advantage of providing a homogeneous source of stimulating Fc fragment. Statistical comparisons of plaque-forming cells (PFC) in the presence and absence of C3a were performed by using Student's *t*-test. In certain experiments, PBL were first pretreated with C3a (10  $\mu\text{g}/\text{ml}$ ) in the presence of 2MGP inhibitor (1 mM) for 1 hr at  $37^\circ\text{C}$ , and then the cells were rinsed (to remove C3a and 2MGP) and were added to an equal number of SGL from the same patient. The cells were then cultured for 6 days in the presence of Fc fragments (1  $\mu\text{g}/\text{ml}$ ), as described above.

The percent suppression induced by C3a, a known inducer of T suppressor cell activity (14), was calculated as follows:

Percent suppression

$$= 100 - 100 \times \frac{(\text{PFC in presence of Fc plus C3a}) - (\text{PFC in absence of Fc and C3a})}{(\text{PFC in presence of Fc}) - (\text{PFC in absence of Fc and C3a})}$$

## RESULTS

**Decreased content of Leu-7<sup>+</sup> cells in SGL.** By using monoclonal antibodies and flow cytometry, we first compared the proportion of lymphocyte subsets in the SGL

and PBL of SS patients. The majority of cells were T cells (Leu-4<sup>+</sup>) of the Leu-3a (T helper) subset in both SGL and PBL (Table I). However, SGL lacked lymphocytes reactive with monoclonal antibodies anti-Leu-7 and anti-Leu-11 that were present in the PBL of the same patients. To confirm these results, we found that SGL also lacked reactivity with monoclonal antibody F45 (which detects a distinct epitope on the Leu-7 antigen). Also, SGL had a significantly lower proportion of cells reactive with monoclonal antibody OKM1 ( $p < 0.05$ ), a marker frequently found on Leu-7<sup>+</sup> and Leu-11<sup>+</sup> cells (20). In agreement with previous reports (6, 7), a B cell subset defined by monoclonal antibody B532 was present in SGL but not in PBL (Table I). Thus, SGL possess certain subsets not found in PBL (i.e., B532<sup>+</sup> cells), but also lacks certain subsets (i.e., anti-Leu-7<sup>+</sup> and anti-Leu-11<sup>+</sup>) present in blood.

Next, we compared the SGL of SS patients to the LN of immunologically normal individuals. The proportions of T cells (Leu-4<sup>+</sup>), T cell subsets (Leu-2a<sup>+</sup> and Leu-3a<sup>+</sup>), and B cells in SGL were not significantly different from those in normal LN (Table I). However, normal LN contained significantly more Leu-7<sup>+</sup> cells than were found in SGL ( $p < 0.05$ ) (Table I). Leu-11<sup>+</sup> cells (a marker of large granular lymphocytes) and OKT6<sup>+</sup> cells (a marker of immature T cells) were not found in either LN or SGL.

To determine whether anti-Leu-7<sup>+</sup> cells could enter salivary gland tissue in patients with nonimmunologic conditions, salivary gland biopsies from patients with salivary gland adenomas were examined, because such biopsies often show extensive lymphocytic infiltrates around the tumor margin (31). Eluates of these tumors, obtained by collagenase digestion (32), contained Leu-7<sup>+</sup> cells (16% ± 7) and Leu-11<sup>+</sup> cells (7% ± 4). These data indicate that these lymphoid subsets are capable of entering the salivary gland under certain circumstances.

**Two-color immunofluorescent characterization of Leu-7<sup>+</sup> cells in PBL and normal LNL.** In view of the significant differences in the proportions of Leu-7<sup>+</sup> cells in SS SGL and normal LN, we additionally investigated the phenotype of Leu-7<sup>+</sup> cells by using two-color immunofluorescent staining (Table II). First, we compared the PBL and SGL from SS patients. In the PBL of primary SS patients, the Leu-7<sup>+</sup> marker was not restricted to a single subset because a) this marker was found on both T cells and non T cells, b) both "helper" and "suppressor" T cell

TABLE II

| Two-color immunofluorescent staining of lymphocyte subsets |                             |                         |                             |                         |
|--|-----------------------------|-------------------------|-----------------------------|-------------------------|
| Phenotypes   | Primary SS PBL <sup>a</sup> | Normal PBL <sup>a</sup> | Primary SS SGL <sup>a</sup> | Normal LNL <sup>a</sup> |
| Leu-7 <sup>+</sup> -Leu-4 <sup>+</sup>                     | 13 ± 5                      | 7 ± 5                   | <2                          | 4 ± 3                   |
| Leu-7 <sup>+</sup> -Leu-4 <sup>-</sup>                     | 10 ± 4                      | 11 ± 6                  | <2                          | <2                      |
| Leu-7 <sup>+</sup> -Leu-2 <sup>+</sup>                     | 12 ± 3                      | 7 ± 6                   | <2                          | <2                      |
| Leu-7 <sup>+</sup> -Leu-3 <sup>+</sup>                     | 4 ± 2                       | <2                      | <2                          | 3 ± 2                   |
| Leu-7 <sup>+</sup> -Leu-11 <sup>+</sup>                    | 7 ± 4                       | 9 ± 3                   | <2                          | <2                      |
| Leu-11 <sup>+</sup> -Leu-4 <sup>+</sup>                    | <2                          | <2                      | <2                          | <2                      |
| Leu-2 <sup>+</sup> -Leu-3 <sup>+</sup>                     | <2                          | <2                      | <2                          | <2                      |
| Isotype control  | <2                          | <2                      | <2                          | <2                      |

<sup>a</sup> The proportions of cells reactive in two-color immunofluorescence were determined by flow cytometry, and were confirmed by fluorescent nucleoscopy for SS PBL (six cases), normal PBL (six cases), SS SGL (six cases), and normal LNL (four cases).

subsets expressed Leu-7; and c) only a subpopulation of Leu-7<sup>+</sup> cells expressed Leu-11 antigen (7% Leu-7<sup>+</sup>-Leu-11<sup>+</sup> and 14% Leu-7<sup>+</sup>-Leu-11<sup>-</sup>). Consistent with our results in Table I, SGL of SS patients did not contain Leu-7- or Leu-11-reactive lymphocytes (Table II). Examples of two-color staining of PBL and SGL from the same SS patient are shown in Figure 1. A subset of PBL is stained by both anti-Leu-4 and anti-Leu-7 (quadrant II in panel B). In panel D, the SGL react with anti-Leu-4 but not with anti-Leu-7. Panels A and C show the contour profiles obtained when PBL and SGL, respectively, stained with control antibodies.

Next, we compared SS PBL to normal PBL and to normal LN. A lower proportion of Leu-7<sup>+</sup>-Leu-3<sup>+</sup> cells was present in the normal PBL than in the SS PBL (Table II) ( $p < 0.05$ ). In normal LNL, a small number of Leu-7<sup>+</sup> cells was noted, and these were predominantly Leu-3<sup>+</sup> (Table II). Examples of two-color staining of normal LN are presented in Figure 2. Panels A and B show the presence of Leu-7<sup>+</sup>-Leu-4<sup>+</sup> and Leu-7<sup>+</sup>-Leu-3<sup>+</sup> cells, respectively. No significant staining of Leu-7<sup>+</sup> cells was noted with anti-Leu-2 (panel C) or control antibodies (panel D). Other staining combinations with the use of lymphoid tissues from normals or primary SS patients demonstrated few (<2%) Leu-2<sup>+</sup>-Leu-3<sup>+</sup> or Leu-4<sup>+</sup>-Leu-11<sup>+</sup> cells. In summary, the results of two-color staining experiments confirmed the deficiency of Leu-7<sup>+</sup> cells in SS SGL as compared with their blood. It is noteworthy that the predominant subset of Leu-7<sup>+</sup> cells in normal LN expressed Leu-3 in contrast to the predominance of Leu-7<sup>+</sup>-Leu-2<sup>+</sup> cells in PBL.

**Immunohistologic location of Leu-7<sup>+</sup> cells.** Although Leu-7<sup>+</sup> cells were not present in suspensions of lymphocytes from the salivary glands of SS patients, it remained possible that such cells were present but were not eluted from the gland. Therefore, frozen tissue sections of salivary gland biopsies were stained by using immunoperoxidase techniques to examine the lymphocyte subsets present in situ. The predominant cell type was the Leu-4<sup>+</sup> T cell (Figure 3, panel A), although B cells (Figure 3, frame B) were present. No staining was noted with antibody anti-Leu-7 (panel C), control myeloma protein (panel D), or antibody anti-Leu-11 (data not shown).

Leu-7<sup>+</sup> cells were present in normal LN and were located predominantly in the germinal center (Figure 4, panels C and D). The majority of T cells were located between germinal centers, but some T cells were also present within these structures (arrows in Figure 4, Panel A). These data and the two-color immunofluores-

TABLE I

Phenotype of lymphocytes defined by monoclonal antibodies

|                         | SS SGL <sup>a</sup> | Normal LNL <sup>a</sup> | SS PBL <sup>a</sup> | Normal PBL <sup>a</sup> |
|-------------------------|---------------------|-------------------------|---------------------|-------------------------|
| Leu-4 (T cells)         | 76 ± 7              | 72 ± 6                  | 75 ± 6              | 71 ± 5                  |
| Leu-3 (T helper)        | 61 ± 5              | 57 ± 6                  | 56 ± 8              | 50 ± 6                  |
| Leu-2 (T suppressor)    | 12 ± 4 <sup>b</sup> | 18 ± 7                  | 24 ± 7              | 31 ± 4                  |
| Leu-12 (B cells)        | 14 ± 5              | 16 ± 4                  | 11 ± 4              | 9 ± 6                   |
| B532 (B cell subset)    | 8 ± 3               | 9 ± 3                   | <1                  | <1                      |
| Leu-7 (HNK-1)           | <1 <sup>b</sup>     | 4 ± 2                   | 19 ± 3              | 17 ± 8                  |
| F45                     | <1 <sup>b</sup>     | 5 ± 2                   | 18 ± 4              | 18 ± 7                  |
| Leu-11 (anti-FcR)       | <1 <sup>b</sup>     | <1                      | 8 ± 5               | 12 ± 3                  |
| OKM1 (anti-C3bIR)       | 7 ± 3 <sup>b</sup>  | 12 ± 3                  | 23 ± 6              | 18 ± 6                  |
| OKT6 (immature T cells) | <1                  | <1                      | <1                  | <1                      |

<sup>a</sup> Percent of reactive lymphoid cells was determined by indirect fluorescence and flow cytometry for SS SGL (six cases), SS PBL (15 cases), normal LN (10 cases), and normal PBL (15 cases).

<sup>b</sup> The proportions of cells stained with monoclonal antibodies Leu-7, F45 and OKM1 in SGL were significantly ( $p < 0.05$ ) lower than in SS PBL, normal LN, or normal PBL. Leu-2<sup>+</sup> T cells in SGL were significantly ( $p < 0.05$ ) lower than SS PBL or normal PBL. The proportion of Leu-11<sup>+</sup> cells in SGL was lower than in PBL from SS patients or normal controls.

Figure 1. Two-color immunofluorescence analysis of PBL and SGL from primary SS patients. PBL and SGL from the same SS patient were stained with fluorescein (Fl)- or phycoerythrin (PE)-conjugated monoclonal antibodies, and were analyzed as described in *Materials and Methods*. PBL (panel B) and SGL (panel D) were stained with Fl-anti-Leu-7 and PE-anti-Leu-4. PBL (panel A) and SGL (panel C) were stained with Fl-conjugated mouse IgG1 and PE-conjugated mouse IgM control proteins. In each sample, the green fluorescence (log scale) is shown along the x axis and the red fluorescence of PE (log scale) along the y axis. On the basis of the control sample, the contour plots were divided into quadrants: I, cells with red fluorescence only; II, cells with both red and green fluorescence; III, unstained cells; and IV, cells with green fluorescence only. The number of cells within each of these areas was integrated and the percentage of the total number of cells was determined. In the control samples (panels A and C),  $\geq 95\%$  of the cells were in quadrant III. In panel B there were 37.2% Leu-4<sup>+</sup>7<sup>+</sup>; 10.3% Leu-4<sup>+</sup>7<sup>-</sup>; and 23.8% Leu-4<sup>-</sup>7<sup>+</sup>. In panel D, there were 88.3% Leu-4<sup>+</sup>7<sup>-</sup>; <1% Leu-4<sup>+</sup>7<sup>+</sup>; and <1% Leu-4<sup>-</sup>7<sup>+</sup>.

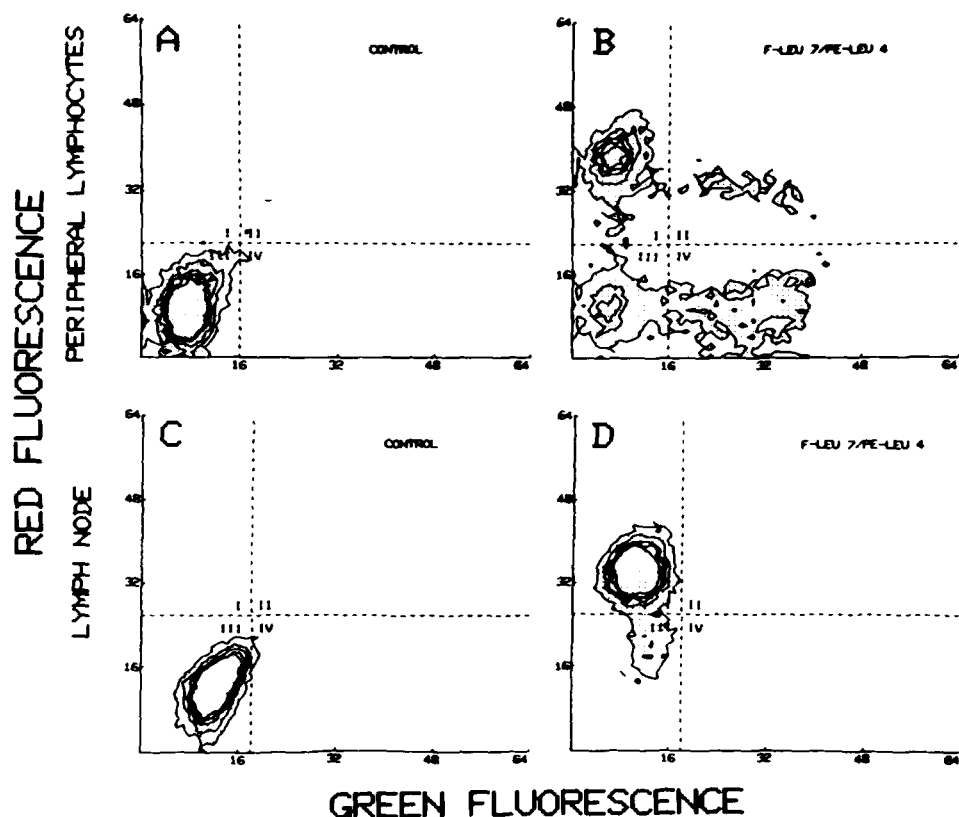
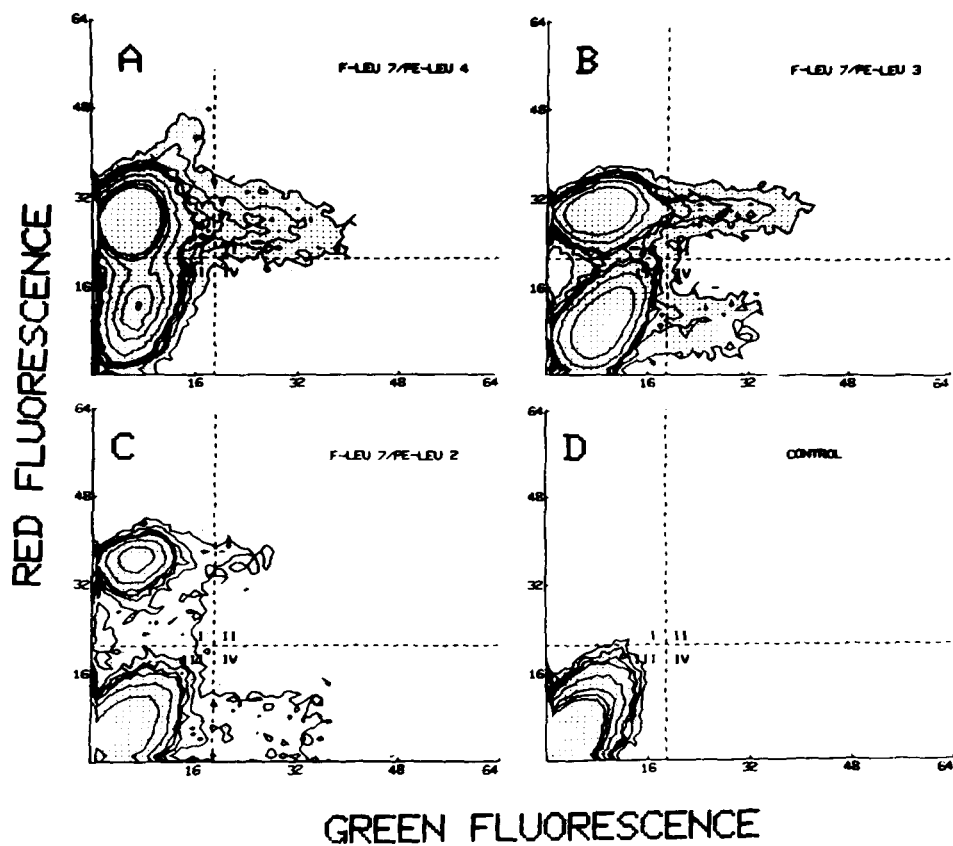


Figure 2. Two-color immunofluorescence analysis of normal LNL. Normal LNL were stained with Fl-Leu-7 and PE-Leu-4 (panel A), Fl-Leu-7 and PE-Leu-3 (panel B), Fl-Leu-7 and PE-Leu-2 (panel C), and Fl-IgM and PE-IgG control proteins (panel D). Samples were analyzed as described in Figure 1 with Fl-(green fluorescence, log scale) shown along the x axis and PE (red fluorescence, log scale) along the y axis. In the control sample (panel D), 99% of the cells were in quadrant III.

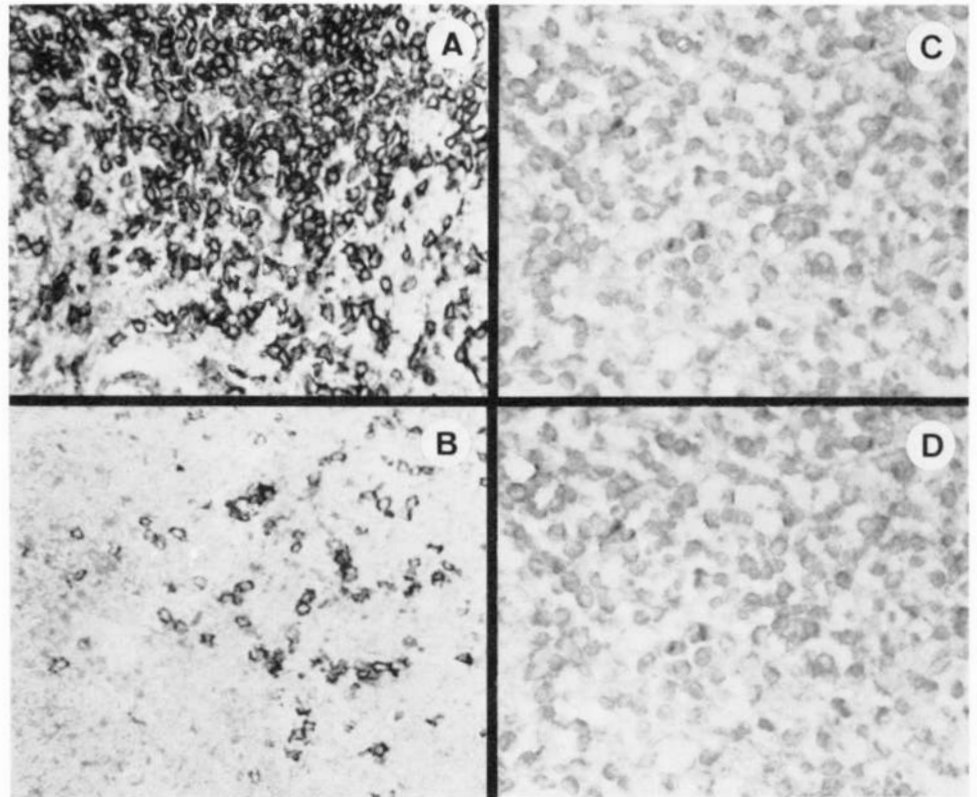


cent staining results (above) suggest that at least some of the Leu-4<sup>+</sup> cells in the germinal center also express Leu-7.

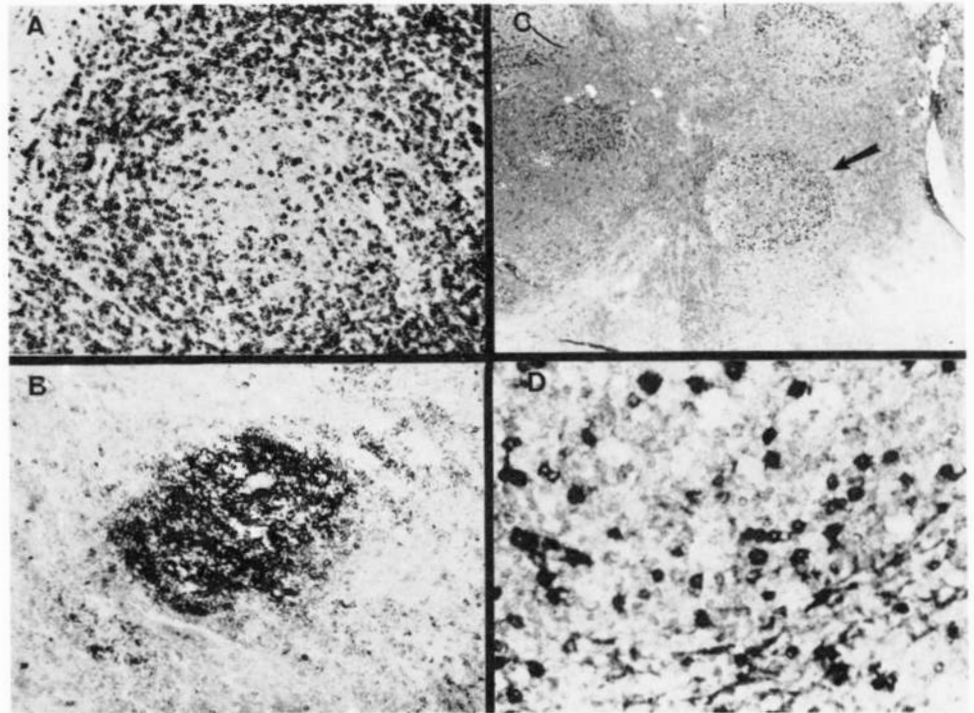
**Decreased NK activity in SGL of primary SS patients.** NK activity was measured by the lysis of <sup>51</sup>Cr-labeled K562 cells. The SGL of SS patients had little NK activity

as compared with the PBL of same patients (Table III). Normal PBL had marginally more NK activity than the PBL of primary SS patients, confirming the data of Miyasaka et al. (33). Normal LNL had little NK activity. These results are consistent with the low levels of Leu-11<sup>+</sup> cells in SGL and normal LNL.

**Figure 3.** Immunoperoxidase-frozen tissue section of a salivary gland biopsy from a primary SS patient. **A.** The majority of lymphocytes are stained with anti-T cell antibody Leu-4 (original magnification 200 $\times$ ). **B.** B cells bearing B532<sup>+</sup> marker are present, but they are not present in clusters. **C.** Cells reactive with anti-Leu-7 were not detected. **D.** Control myeloma proteins were used in place of specific monoclonal antibodies.



**Figure 4.** Immunoperoxidase-stained frozen tissue section of normal tonsillar LN. **A.** Mature T cells (Leu-4<sup>+</sup>) are found predominantly between the germinal centers, although some positively stained cells within the germinal center are present. **B.** The B532<sup>+</sup> B cell subset is located within the germinal center. Original magnification in **A** and **B** was 200 $\times$ . **C.** Leu-7<sup>+</sup> cells are located within the germinal center designated by arrow (original magnification 50 $\times$ ). **D.** Higher magnification of Leu-7<sup>+</sup> cells in germinal center (original magnification 400 $\times$ ).



*Enhancement of polyclonal antibody synthesis by Fc fragments.* Fc fragments of human IgG stimulate polyclonal antibody synthesis by human PBL (30). Because this may be an immunoregulatory signal in vivo (30), we examined the response of lymphoid tissue from SS patients and normal controls to Fc fragments (Table IV). A variable but definite increase in PFC was noted after normal PBL or LNL were cultured in the presence of Fc fragments for 6 days. Similar but less marked increases in PFC were noted in the SGL and PBL from SS patients.

We have previously demonstrated that optimal production of PFC occurs after 6 to 8 days of culture with Fc fragments for blood and lymphoid tissue (6). To extend these results, we examined the responses to different doses of Fc fragments for the SGL of SS patients and the LNL of normals. An optimal response at 0.1 to 1  $\mu\text{g}/\text{ml}$  was noted (Figure 5) for both cell types, suggesting that the SGL from SS patients were not more sensitive to Fc fragments than the lymphocytes from normals.

*SGL from SS patients are not suppressed by C3a.*

TABLE III

NK activity of lymphoid cells from SS patients and normal controls

|                 | Percent Lysis of<br><sup>51</sup> Cr - K562 Cells |         |
|-----------------|---|---------|
|                 | Effector to<br>target ratio                       |         |
|                 | 20:1  | 60:1    |
| SS              |   |         |
| SGL (n = 6)     | 4 ± 3   | 7 ± 4   |
| PBL (n = 6)     | 21 ± 6  | 52 ± 10 |
| Normal controls |   |         |
| LNL (n = 5)     | 5 ± 3   | 9 ± 3   |
| PBL (n = 6)     | 27 ± 8  | 59 ± 12 |

TABLE IV

Stimulation of lymphoid tissue from normals and SS patients by Fc fragments of human IgG

|                            | Fc-Frag-<br>ments <sup>a</sup> | PBL<br>(PFC/10 <sup>6</sup> Cells) | LNL<br>(PFC/10 <sup>6</sup> Cells) |
|----------------------------|--------------------------------|------------------------------------|------------------------------------|
| Normal lymphoid tissue     |                                |                                    |                                    |
| Case 1                     | -                              | 25 ± 8                             | 625 ± 42                           |
|                            | +                              | 492 ± 17                           | 2884 ± 387                         |
| Case 2                     | -                              | 334 ± 34                           | 783 ± 121                          |
|                            | +                              | 1633 ± 202                         | 2607 ± 34                          |
| Case 3                     | -                              | 59 ± 42                            | 33 ± 5                             |
|                            | +                              | 567 ± 101                          | 2975 ± 23                          |
| Case 4                     | -                              | 104 ± 42                           | 18 ± 5                             |
|                            | +                              | 560 ± 59                           | 930 ± 22                           |
| Case 5                     | -                              | 35 ± 15                            | 92 ± 9                             |
|                            | +                              | 2180 ± 60                          | 467 ± 31                           |
| Primary SS lymphoid tissue |                                |                                    |                                    |
| Patient 1                  | -                              | 95 ± 13                            | 290 ± 12                           |
|                            | +                              | 450 ± 91                           | 1530 ± 42                          |
| Patient 2                  | -                              | 56 ± 18                            | 95 ± 13                            |
|                            | +                              | 344 ± 42                           | 450 ± 91                           |
| Patient 3                  | -                              | 80 ± 12                            | 120 ± 10                           |
|                            | +                              | 272 ± 12                           | 420 ± 25                           |
| Patient 4                  | -                              | 57 ± 12                            | 80 ± 12                            |
|                            | +                              | 65 ± 15                            | 272 ± 14                           |

<sup>a</sup> Fc fragments at 1 μg/ml. Cell cultures were performed in quadruplicate.

Complement fragment C3a inhibits polyclonal activation of normal human PBL induced by Fc fragments (14). Our data indicate that C3a also inhibits normal human LNL and PBL from SS patients (Table V). However, considerably less inhibition of SS SGL by C3a was noted ( $p < 0.05$ ). Levels of C3a as high as 50 μg/ml failed to suppress Fc-induced stimulation of the SGL.

In SS patients nos. 2 and 3, T cells from peripheral blood (that contained 18 and 22% F45<sup>+</sup> cells, respectively) were prepared. These T cells were exposed to C3a in the presence of 2MPG, were rinsed, and were added to the autologous SGL that had been stimulated by Fc fragments. The polyclonal response of the SGL was inhibited by >80% by the C3a activated autologous T cells. These results demonstrate that SGL B cells are sensitive to down regulation by T cells exposed to C3a.

## DISCUSSION

Patients with SS provide an unusual opportunity to characterize lymphocyte subsets present at the site of autoimmune tissue destruction (i.e., the salivary glands). Previous studies have shown that SGL contain lymphoid subsets (i.e., B532<sup>+</sup> B cells) not present in the same patient's PBL. This study extends those findings by demonstrating that SGL lack certain lymphoid subsets (defined by antibodies anti-Leu-7 and anti-Leu-11) that are present in their blood. The absence of this subset could not be explained by the loss of the Leu-7 epitope from the

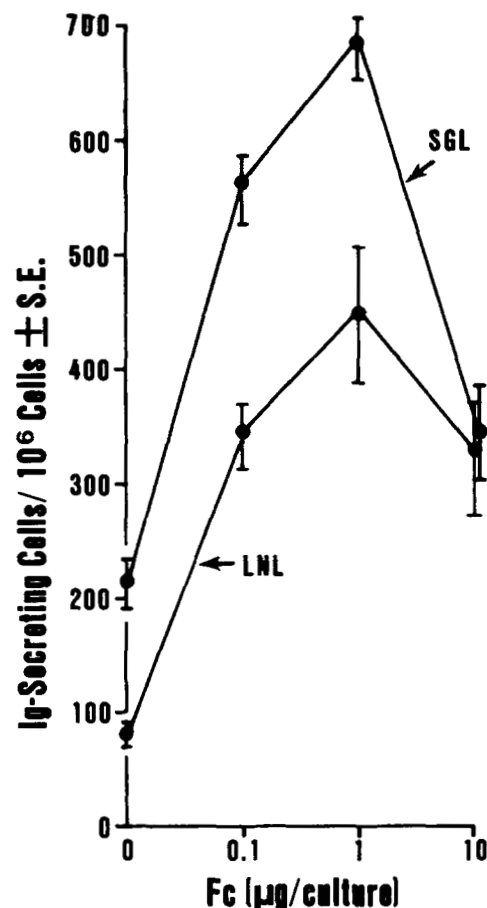


Figure 5. Dose response of Fc fragment-mediated polyclonal antibody response by LNL of immunologically normal adults or SGL of primary SS patients. The number of PFC at day 6 of culture was measured by using the protein A reverse plaque assay with the use of amplifying sera that detect IgG + IgA + IgM. The curves show representative results from LNL (one normal) and from SGL (one SS patient). Similar curves were obtained with SGL from three different SS patients and with LNL from three normal individuals, showing that optimal production occurred at 0.1 to 1 μg/ml of Fc fragments.

TABLE V

Suppression of polyclonal B cell activation by complement C3a<sup>a</sup>

|                            | Percent Suppression<br>by PFC |
|----------------------------|-------------------------------|
| Normal LN                  |                               |
| Case 1                     | 69                            |
| Case 2                     | 81                            |
| Case 3                     | 73                            |
| Case 4                     | 86                            |
| Case 5                     | 72                            |
| SGL of primary SS patients |                               |
| Patient 1                  | 26                            |
| Patient 2                  | (0)                           |
| Patient 3                  | 10                            |
| Patient 4                  | 14                            |

<sup>a</sup> Complement C3a at 10 μg/ml and carboxypeptidase inhibitor (2MPG) at 1 mM. Fc fragments at 1 μg/ml. All cell cultures were performed in quadruplicate.

SGL, because these cells also failed to react with monoclonal antibody F45, an antibody that detects a distinct epitope on the same antigen. Furthermore, it is unlikely that our failure to detect the Leu-7 or Leu-11 subsets could be ascribed to "masking" of the epitopes. Incubation of cell suspensions at 37°C for 2 hr failed to reveal cryptic determinants, and evaluation of tissue sections for cytoplasmic antigen proved negative. Thus, Leu-7<sup>+</sup> and Leu-11<sup>+</sup> cells were present in PBL of SS patients but absent from their SGL.

This study also demonstrates that the lymphocyte subsets defined by monoclonal antibodies anti-Leu-4 (total T cells), Leu-3a (T helper), and Leu-2a (T suppressor) in the SGL of SS patients were similar to those found in normal tonsillar LN. However, the SGL differed significantly from normal LN in their proportion of Leu-7<sup>+</sup> cells, suggesting an important difference between lymphoid infiltrates in patients with SS and in immunologically normal individuals. The localization of different lymphocyte subsets within lymphoid tissues is probably governed by dendritic cells (34, 35). Thus, B cells and Leu-7<sup>+</sup> cells are organized into germinal center regions around "follicular" dendritic cells, whereas T cells (but not Leu-7<sup>+</sup> cells) are organized around "paracortical" dendritic cells (13, 36). Therefore, we propose that the salivary gland lesions in SS contain predominantly "paracortical" dendritic type cells, and that this accounts for the predominantly T cell infiltrate with an absence of Leu-7<sup>+</sup> cells. According to this model, Leu-7<sup>+</sup> would be present in the patient's circulation but would not be able to adequately localize (or "home") to the salivary gland lesions. In support of this hypothesis, we have found that frozen tissue sections of salivary glands from SS patients contain numerous dendritic cells that can be identified by anti-HLA-DR antibodies (3, 6, 7, 22). However, these biopsies usually contain few (or no) dendritic cells bearing complement C3b receptors (unpublished observations), a marker found on "follicular" dendritic cells (36).

Finally, we wished to determine whether the absence of Leu-7<sup>+</sup> cells could influence the functional properties of SGL from SS patients. We chose an *in vitro* system recently described by Morgan et al. (14) in which polyclonal B cell activity could be inhibited by complement fragment C3a, because studies on normal PBL and LN suggested that Leu-7<sup>+</sup> cells were required for inhibition (unpublished observations). These results demonstrated that SGL were not inhibited by C3a, in contrast to normal LN lymphocytes. Leu-7<sup>+</sup> cells in peripheral blood have previously been shown to have "suppressor" cell properties after exposure to immune complexes (37) or interferon- $\gamma$  (26, 38). It is likely that complement C3a represents another method of activating Leu-7 cells to this activity. Immune complexes, interferon- $\gamma$ , and complement activation products are probably present at sites of autoimmune tissue destruction (2, 4, 39). Thus, the absence of Leu-7<sup>+</sup> cells at this site may remove an important immunoregulatory circuit that is present in normal lymphoid tissue.

In summary, these results have extended previous studies that demonstrate phenotypic and functional differences between inflammatory sites and blood in the same patient. First, these results emphasize that caution must be used when theories of pathogenesis are based exclusively on abnormalities found in blood lymphocytes. Secondly, we found that the SGL of SS patients differed from normal tonsillar lymphocytes in their content of Leu-7<sup>+</sup> cells. Although the precise function of Leu-7<sup>+</sup> cells *in vivo* remain unknown, they can be stimulated *in vitro* to suppress polyclonal B cell activation. Thus, the absence of such cells in the salivary glands of SS patients may remove an important immunoregulatory circuit. These results also point out that the suppressor cells induced by C3a are distinct from NK cells (i.e., cells defined by the ability to lyse K562 target cells). The

suppressor cells have the phenotype Leu-7<sup>+</sup>-Leu-11<sup>-</sup>, whereas the NK cell activity is predominantly in the Leu-11<sup>+</sup> subset (20).

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