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LYMPHOCYTE SUBSETS IN SJOGREN'S SYNDROME: A QUANTITATIVE ANALYSIS USING MONOCLONAL ANTIBODIES AND THE FLUORESCENCE-ACTIVATED CELL SORTER

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SUMMARY Lymphocyte subsets in the peripheral blood of 18 patients with Sjogren's syndrome (SS) were studied using monoclonal antibodies and the fluorescence-activated cell sorter (FACS). The percentage of T cells was decreased when compared to normal controls. In primary SS, there was a proportional decrease in both suppressor/cytotoxic (anti-Leu-2a reactive) and helper/inducer (anti-Leu-3a reactive) T cells with an unchanged helper/suppressor ratio (1.8 vs. 1.7 for normals). In SS with an associated connective tissue disorder, there was a significant decrease only in the suppressor/cytotoxic subset. There was increase in B cells and null cells in primary SS compared to controls.

Quantitative immunofluorescence allowed the calculation of determinant density per cell. Cells expressing low antigen density Leu-2a were increased in 8 patients (4 with primary SS and 4 with SS with an associated disorder). Thus, in addition to quantitative changes in lymphocyte subsets, we found changes in Leu-2a expression suggesting abnormal differentiation of the suppressor/cytotoxic subset. These changes may contribute to the immunoregulatory disturbance in Sjogren's syndrome.

INTRODUCTION

SJOGREN's syndrome (SS) is a chronic autoimmune disease characterized by lymphoid cell infiltration of exocrine glands, especially lacrimal and salivary glands, resulting in xerophthalmia and xerostomia (1). Some patients manifest lymphocyte aggressive behavior with extraglandular lymphocytic infiltration and, rarely, progression to malignant lymphoma or Waldenstrom's macroglobulinemia (2, 3, 4). Primary SS (sicca complex unaccompanied by an associated disorder) may be distinguished from secondary SS (SS occurring with rheumatoid arthritis) clinically, immunogenetically and serologically (5, 6).

Patients with SS have features suggestive of B cell hyperactivity. These include polyclonal hypergammaglobulinemia, the presence of various autoantibodies (such as rheumatoid factor and anti-nuclear antibodies) and circulating immune complexes (7, 8, 9). Evidence for abnormal cellular immunity and disordered immuno-

regulation includes impaired delayed-type skin reactions, decreased response to mitogens (10, 11) and a decreased autologous mixed lymphocyte reaction (AMLR) (12).

Alterations in lymphocyte subsets in multiple sclerosis (13) and in systemic lupus erythematosus (SLE) (14) have been detected using monoclonal antibodies. We have performed similar studies in 9 patients with primary SS and 9 patients with SS plus an associated connective tissue disease. For this analysis, we used monoclonal antibodies to the cell-surface antigens of helper (anti-Leu-3a) and suppressor (anti-Leu-2a, anti-Leu-2b) T cell subsets and to common T cell antigens (anti-Leu-1, anti-Leu-4) (15). The Leu reagents identify the same T cell subsets as the OKT series of monoclonal antibodies (16). Anti-Leu-3a is analogous to OKT4, anti-Leu-2a/2b to OKT8, anti-Leu-1 to OKT1, and anti-Leu-4 to OKT3. Monoclonal anti-HLA-DR and goat anti-human Ig were used to identify B cells. In this study, we found quantitative changes in T, B and null cell proportions. In addition, by using quantitative immunofluorescence, we extend the use of monoclonal antibodies and the FACS in the study of human disease. We report decreased Leu-2a antigen expression suggesting abnormal differen-

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tiation of the suppressor/cytotoxic T cell subset. These changes may contribute to the immunoregulatory disturbances in SS.

MATERIALS AND METHODS

Patients

Eighteen patients seen in the Sjogren's Syndrome Clinic at the University of California, San Francisco, were studied. There were 5 males and 13 females, ages 30 to 75 years (average 45 years). SS was diagnosed according to previously established criteria (17). Keratoconjunctivitis sicca was diagnosed after complete ophthalmologic evaluation including Rose-Bengal and fluorescein staining and the Schirmer test (18). Xerostomia was established by a reduction in the stimulated parotid flow rate and a focus score of more than 1 on labial salivary gland biopsy (19). Rheumatoid arthritis (RA) and SLE were diagnosed by standard diagnostic criteria (20, 21). Nine SS patients had only glandular (primary SS) and another 9 had SS with an associated connective tissue disease (3 with SLE, 2 with RA and 4 with scleroderma). Four patients (3 with SLE and 1 with RA) were on Prednisone (less than 10 mg/day). The normal group consisted of 5 males and 5 females, ages 29 to 55 years (average 37 years).

Isolation of Lymphocytes

Peripheral blood mononuclear cells were isolated from heparinized venous blood by density centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) as previously described (12). The mononuclear cells were collected from the interface, washed three times, and resuspended in complete RPMI-1640 containing 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES supplemented with 1% fetal calf serum and 0.01% sodium azide.

Monoclonal Antibodies Used to Detect Human Lymphocyte Antigens

Eight monoclonal antibodies were used in the present study. Fluorescein conjugated anti-Leu-1, anti-Leu-2a, anti-Leu-2b, anti-Leu-3a, anti-Leu-4, anti-Leu-5 and anti-HLA-DR (Becton-Dickinson, FACS systems, Monoclonal Antibody Center, Sunnyvale, Ca.) were used to obtain density-distribution curves (FACS profiles). OKM1 (Ortho Pharmaceuticals, Raritan, N.J.) was used with a fluorescein-conjugated goat anti-mouse IgG as a second step. Fluorescein conjugated goat F(ab)₂ anti-human immunoglobulin (Tago Inc., Burlingame, Ca.) was used to directly stain cells with surface immunoglobulin. The preparation and characterization of these antibodies has been previously described (15, 22, 23, 24, 25).

In brief, anti-Leu-1 reacts with more than 95% of peripheral T cells and anti-Leu-4 with 80–95% of T cells (15). The Leu-5 antigen is either identical to or closely associated with the human T lymphocyte receptor for sheep erythrocytes (SRBC) (26). Leu-2a/Leu-2b and Leu-3a are expressed on different T lymphocyte subsets identifying either suppressor/cytotoxic or helper/inducer T cells (15, 23). Anti-HLA-DR reacts with human B lymphocytes, monocytes/macrophages and activated-T cells (24). OKM1 reacts with 78% of peripheral blood adherent cells, granulocytes and approximately 18% of small non-adherent Ia⁻ cells (25).

Immunofluorescent Staining and FACS Analysis

Direct immunofluorescent staining of mononuclear cells were performed using fluorescein-conjugated antibodies (except for OKM1). Reagents were first centrifuged at 100,000 g for 10 min. One million lymphoid cells per well in microtiter plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.) were mixed with saturation levels of the reagents on ice for 30 min in the presence of 0.01% sodium azide. For OKM1 staining, after washing, a second step of fluorescein-conjugated goat anti-mouse IgG was used. Light scatter windows were set to include only live lymphocytes.

Ten thousand viable lymphocytes were analyzed on a fluorescence-

activated cell sorter (FACS II, B-D FACS Systems, Mountain View, Ca.) and the percentage of stained cells was determined by integration from the point where fluorescent intensity was greater than the (auto) fluorescence of unstained cells (27).

Quantitative fluorescence measurements were made on a modified FACS II fitted with a logarithmic amplifier that gave the fluorescence intensity values distributed over a 10⁴ range. The number of antibody molecules bound to positive cells was determined, as previously described using the following calculation:

$$\frac{\text{mean FE (positive cells)} - \text{mean FE (negative cells)}}{\text{fluorescein : protein (F/P) ratio of purified antibody}}$$

FE = fluorescein equivalents.

Fluorescein equivalents were determined after calibration of the FACS with fluorescein solutions of known concentration.

Mitogen Stimulation

2 × 10⁵ mononuclear cells in 200 µl of complete RPMI-1640 with 20% fetal calf serum (FCS) were stimulated either with 1 µg of purified phytohemagglutinin (PHA) (Burroughs-Wellcome & Co., Reagent Div., Greenville, N.C.), or 20 µg of Concanavalin A (Con A) (Miller Laboratories, Elkhart, In.), or 20 µl (1:10 dilution) of pokeweed mitogen (PWM) (Gibco Laboratories) for 72 hr at 37°C in 5% CO₂. These doses of mitogen were shown to be optimal for maximal stimulation. Cultures were performed in triplicate. Cultures were pulsed with 1 µCi/well of tritiated thymidine (methyl-³H) TdR, specific activity 6.0 Ci/mmol (Schwarz-Mann, Division of Becton-Dickinson & Co., Orange, N.Y.), 20 hr before harvesting. The data are expressed as differences in counts per minute (cpm) for cultures containing mitogen and cpm for cultures without mitogen (Δ cpm).

Detection of Antilymphocyte Antibody

The modified Terasaki method was used as described (12). Briefly, 2 × 10⁵ mononuclear cells in 50 µl were incubated with 50 µl of serum at 15°C for 30 min followed by 50 µl of rabbit complement at 15°C for 180 min. The viability of cells was measured by trypan blue exclusion. Serum that killed more than 20% cells was considered positive for cytotoxicity.

Statistical Analysis

Data were analyzed with the Student's *t*-test. Results are expressed as mean ± standard error of the mean (SEM).

RESULTS

Reactivity with Monoclonal Anti-T Cell Antibodies

The reactivity of peripheral blood lymphocytes with monoclonal antibodies to T cell surface antigens is presented in Table 1. In normal subjects 73.6 ± 2.6% of lymphocytes were T cells on the basis of reactivity with anti-Leu-1 or anti-Leu-4. Anti-Leu-2a, which defines the suppressor/cytotoxic T cell subset, stained 28.5 ± 2.6% of PBL while anti-Leu-3a, which defines the helper/inducer subset, stained 44.6 ± 2.6% of PBL. The normal ratio of Leu-3a/Leu-2a was 1.7 ± 0.2. In primary SS, there was a significant decrease in the percentage of lymphocytes reactive with anti-Leu-1, i.e. total T cells (56.1 ± 5%). Both suppressor and helper-T cell subsets were diminished with unchanged ratio of Leu-3a/Leu-2a (1.82 ± 6.3).

In SS with associated disease there was a less dramatic but significant decrease in the percentage of T cells (62.8 ± 4.5% reactive with anti-Leu-1). This was due

Table 1 Lymphocyte proportions in patients with SS and normal controls*

Disease category	Percentage of lymphocytes reactive with monoclonal antibodies					Goat F(ab) ₂ anti-human Ig†	Ratio of Leu-3a/Leu-2a
	Goat F(ab) anti-human Ig†	Anti-Leu-1	Anti-Leu-2a	Anti-Leu-3a	Anti-HLA-DR		
Normal	10	73.6 ± 2.6	28.5 ± 2.6	44.6 ± 2.6	10.9 ± 0.8	25.8 ± 0.8	1.7 ± 0.2
Primary SS	9	56.1 ± 4.9‡	21.9 ± 3.1	35.3 ± 4.0‡	14.2 ± 1.3‡	36.9 ± 4.4‡	1.8 ± 0.2
SS with assoc.	9	62.8 ± 4.5‡	21.2 ± 2.3‡	40.8 ± 4.6	14.0 ± 1.6	30.7 ± 3.1	2.2 ± 0.4

* Results are expressed as mean ± S.E.M.

† The percentages given are the sum of the percentages of the dull and the bright peak of positive cells. The bright cells correspond to the DR⁺ cells and the dull cells are primarily non-T, non-B cells.

‡ Significant differences ($p < 0.05$) based on 95% of confidence limits between patients and normal controls.

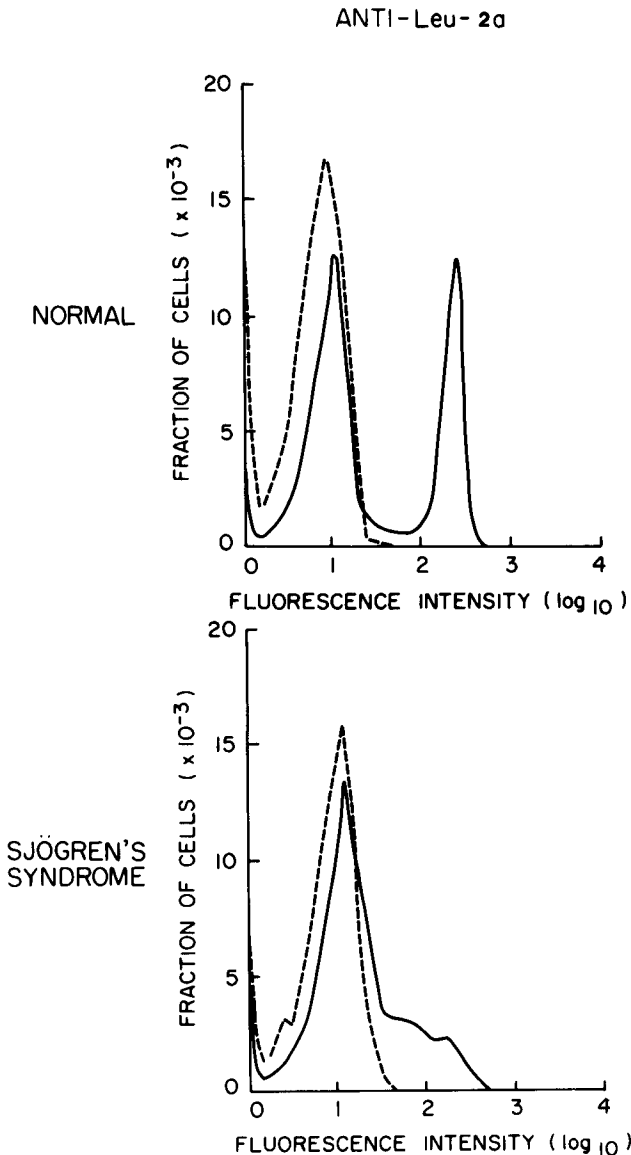


FIG. 1. FACS analysis of Leu-2a determinant expression on PBL from a patient with SS and from a normal control. Directly conjugated anti-Leu-2a was used to stain PBL. Light scatter windows were set to include only live lymphocytes. Autofluorescence signals appear as a peak on the left of the profiles extending approximately to 1.2 (\log_{10}) fluorescence intensity units (dotted line).

primarily to a decrease in the proportion of PBL reactive with anti-Leu-2a ($21.2 \pm 2.3\%$).

In 8 of 18 patients there was a decrease, relative to normal subjects, in the Leu-2a antigen density/cell. This decrease was apparent on visual inspection of the FACS profile (fig. 1), and ranged from a 10% to a 50% reduction. For the patient shown, PBL reactive with anti-Leu-2a bound an average of 3×10^4 antibody molecules/cell vs. 6×10^4 antibody molecules/cell in normals. Because directly conjugated antibodies were used, this provides a good measure of relative antigen density/cell. These 8 patients included 4 with primary SS, 2 with associated RA and 1 each with associated SLE and scleroderma. There was no difference in Leu-3a determinant expression in SS.

Neither the change in lymphocyte proportion nor the change in antigen density are explained by blocking serum factors, as shown by the following: (1) anti-lymphocyte antibody was not detected in any of the 18 patients; (2) preincubation of normal PBL with SS serum (from 5 patients with abnormal FACS profiles) did not alter the pattern obtained with anti-Leu-1, anti-Leu-2a and anti-Leu-3a; and (3) the sum of anti-Leu-2a and anti-Leu-3a reactive T cells accounts for the total T cell population as defined by anti-Leu-1 or anti-Leu-4. Thus, it is unlikely that cell surface determinants are blocked by serum factors.

Reactivity with Anti-HLA-DR and Anti-Ig

Anti-HLA-DR reacted with $10.9 \pm 0.8\%$ of PBL in normals, $14.5 \pm 1.3\%$ of PBL in primary SS ($p < 0.05$) and $14.0 \pm 1.6\%$ of PBL in patients with SS and an associated disease ($p > 0.05$). There was no difference in determinant density/cell between the three groups (data not shown).

Goat F(ab)₂ anti-human immunoglobulin reacted with $25.8 \pm 2.5\%$ PBL in normals, with $36.9 \pm 4.4\%$ of PBL in primary SS and with $30.7 \pm 3.1\%$ of PBL in SS with an associated disease. Thus, there was a significant increase in cells detected by anti-human immunoglobulin in primary SS ($p < 0.025$). With the aid of logarithmic amplification, two subpopulations of Ig bearing lympho-

ANTI-Ig

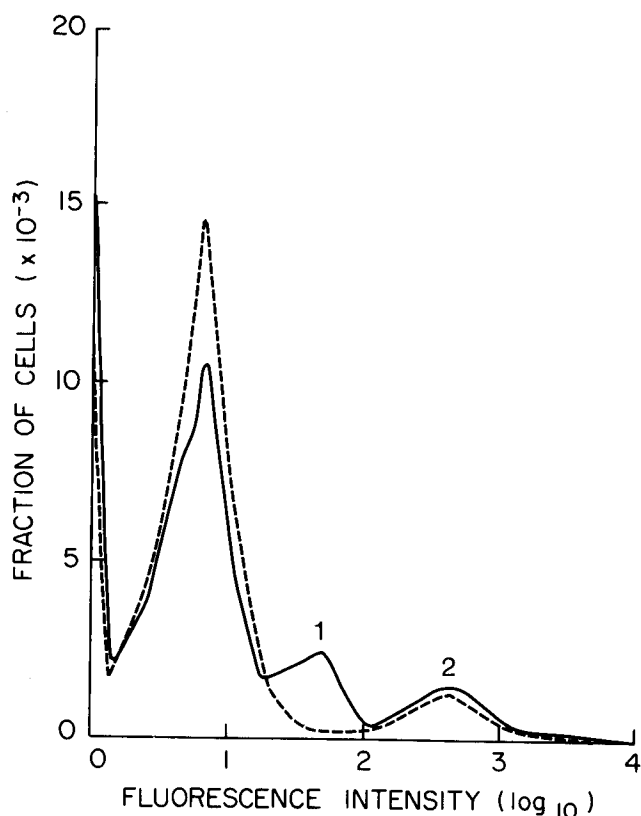


FIG. 2. FACS analysis of Ig⁺ PBL population from a normal control. Directly conjugated goat F(ab')₂ anti-human immunoglobulin was used to stain PBL. Stained PBL were distributed in 2 peaks (1 and 2), while peak 1 represents unstained PBL. After PBL were incubated for 1 hr at 37°C in the absence of human serum, peak 1 had greatly diminished whereas there was no change in peak 2.

cytes were definable (fig. 2) in the majority of patients and controls. This is in accord with the findings of others (29, 30).

Further analysis of these subpopulations revealed that the high antigen (Ig) density subpopulation (peak 2, fig. 2) identified B cells because this percentage was identical to the percentage of cells reactive with anti-HLA-DR. The low antigen density subpopulation (peak 1, fig. 2) represented the binding of autologous Ig to lymphocytes via Fc receptors because this peak disappeared or decreased after incubation of PBL at 37°C for 1 hr in the absence of human serum (dotted line, fig. 2). Additive staining

ANTI-Ig + OKM1

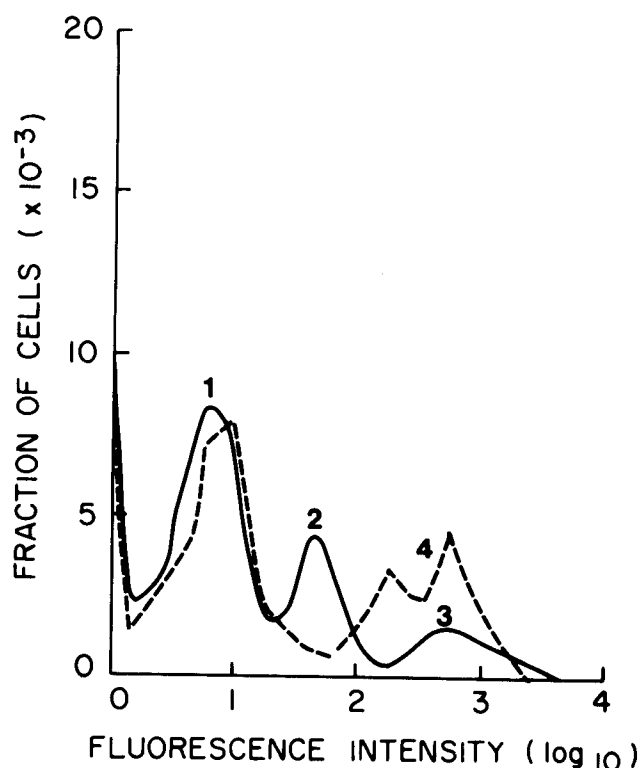


FIG. 3. FACS analysis of Ig⁺ PBL populations from normal control. Directly conjugated goat F(ab')₂ anti-human Ig was used to stain PBL (solid line). Additive staining with anti Ig and OKM1 resulted in a selective increase in staining brightness of cells in peak 2 (peak 4, dotted line).

demonstrated that most of the lymphocytes in peak 2 reacted with OKM1. This is shown by the selective increase in staining brightness of this subpopulation by the inclusion of OKM1 in the staining mixture (peak 4, dotted line, fig. 3). A few of the lymphocytes in peak 2 reacted with anti-Leu-5 but none reacted with anti-Leu-1 or anti-HLA-DR. Thus, these cells represent the Fc receptor bearing, OKM1 positive, Ia and Leu-1 negative subpopulation of null cells.

Mitogen Responses

As shown in Table 2, the response to PHA and Con A was significantly depressed in both SS groups compared to normals. The response to PWM was not altered.

Table 2 Mitogen responses in patients with SS and normal controls*

Disease category	Number of patients	PHA response	Con A response	PWM response
Normal	10	57,539 ± 6,435	56,535 ± 3,886	25,560 ± 4,392
Primary SS	9	34,767 ± 7,162†	28,251 ± 6,654†	21,439 ± 5,550
SS with assoc.	9	32,703 ± 13,079	35,927 ± 8,369†	36,611 ± 5,860

* Results are expressed as mean ± S.E.M.

† Significant differences (p < 0.05) based on 95% confidence limits between patients and normal control groups.

DISCUSSION

Monoclonal antibodies and quantitative immunofluorescence with a FACS were used to characterize lymphocyte subsets in SS. Changes in both lymphocyte proportions and antigen density were found. There was a decrease in the percentage of T cells in primary SS with a proportional decrease in both helper and suppressor subsets. In SS associated with another connective tissue disease, there was a less dramatic decrease in the percentage of T cells but a significant decrease in the suppressor subset.

Leu-2a is expressed, in low density, on virtually all immature thymocytes. When these cells enter the periphery, the Leu-2a antigen increases in density and is restricted to the suppressor/cytotoxic T cell subset (15). Leu-2a antigen on circulating lymphocytes in 8 of 18 SS patients was expressed in low density. This could reflect: (i) genetic polymorphism in the Leu-2a antigen; (ii) abnormal expression of the antigen due to blocking factors in SS serum; or (iii) an abnormality in the differentiation of the suppressor/cytotoxic subset. Genetic polymorphism in the anti-Leu-2a determinant is unlikely because it was not detected in normals. Further, a similar FACS profile was obtained with anti-Leu-2b. It is also unlikely that serum factors blocked Leu-2a expression, as pre-incubation of normal PBL with SS serum did not alter the FACS profile obtained with anti-Leu-2a or anti-Leu-2b. Thus, the change observed probably reflects abnormal differentiation of the suppressor/cytotoxic subset.

Whether this change is directly related to effector function is not clear. However, these molecules probably perform essential functions for the cells on which they are found, as anti-Leu-2 monoclonal antibody blocks killing by human cytotoxic T cells (23). Anti-Leu-2a and anti-Leu-2b are homologous to the mouse Lyt-2 and Lyt-3 antigens respectively (15). Lyt-2 and Lyt-3 are closely linked to the mouse κ chain region on Chromosome 6 (31). These macromolecules are closely associated with, but are probably not the mouse T cell antigen receptor, and are most likely involved in cellular interactions (32). A study of the cells with abnormal expression of these antigens is currently underway in our laboratory and may provide clues to the functional interaction of these molecules and to their role in immunoregulation.

SS encompasses a spectrum from benign lymphoid infiltration to a monoclonal B cell lymphoma (2). Our laboratory has previously reported an increase in B cells in the peripheral blood from patients with SS (33). The present study confirms the increase in B cells in primary SS by using anti-HLA-DR and anti-Ig staining. Moutsopoulos and Fauci (34), on the other hand, reported normal numbers of B and T cells in SS. The conflicting results may reflect differences in technique or differences in patient selection. In fact, we observed a

wide variation in T lymphocyte subsets within each group that was not attributable to technical differences (estimation error at most 1 or 2%). Function studies also support heterogeneity among patients with primary and secondary SS (10, 11).

The increased Ig bearing lymphocytes in primary SS were distributed in two subpopulations. The bright subpopulation consisted of B cells, as the percentage of these cells was identical to the percentage of HLA-DR⁺ cells. The dull subpopulation consisted of null cells binding autologous Ig via their Fc receptors as shown by the disappearance of this subpopulation after incubation of PBL for one hour at 37°C in the absence of human serum. Van Boxel *et al.* (35) reported an increase in B cells carrying more than one heavy chain isotype in SS. Our results suggest that the increase observed could also represent an increase in the null cells binding Ig via their Fc receptors.

The relative increase in non-T, non-B lymphocytes in SS has not been reported, but has been described in the NZB mouse (36) and in patients with SLE (34). Additive staining demonstrated that this dull subpopulation was anti-Leu-1, anti-Leu-4 and anti-HLA-DR nonreactive. Most of the lymphocytes in this subpopulation were OKM1 reactive. This population has the phenotype of the NK/K cell (38, 39). However, NK activity was significantly decreased in both SS groups (40, 41).

A selective decrease in the suppressor T cell subset in patients with active SLE and active multiple sclerosis has been reported (13, 14). In our study, some patients had a relative reduction in the suppressor/cytotoxic T cell subset, but the loss was not as great as reported in active SLE. Functional studies have similarly shown defective Con A-generated suppressor cells in SLE but not in SS (34). Further, spontaneous polyclonally stimulated B cells were demonstrated in SLE but not in SS (42). Functional alterations are more severe in extraglandular SS or SS with an associated connective tissue disease than in primary SS (11, 12). The associated disorder superimposed upon SS probably contributes to the numerical and functional alterations observed.

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