

Decreased Autologous Mixed Lymphocyte Reaction in Sjögren's Syndrome

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ABSTRACT The autologous mixed lymphocyte reaction (AMLR) measures the response of peripheral blood T cells to antigens present on the surface of non-T cells. The AMLR was studied in 25 patients with Sjögren's syndrome (SS). The AMLR was decreased in 15 of 25 (60%) of patients with SS ($5,272 \pm 6,738$ cpm vs. $14,396 \pm 10,092$ cpm for the normal controls, $P < 0.001$). The AMLR was decreased in 8 of 15 patients with only glandular disease who were not on any systemic medications. Patients with SS and associated disease had lower responses than patients with SS alone. Two patients with pseudolymphoma had absent response. The decreased AMLR correlated with a decreased response to concanavalin A, suggesting a possible abnormality of a T cell subpopulation. There was no correlation between the decreased AMLR and age, focus score, serum immunoglobulin concentration, the titer of antilymphocyte antibody, or phytohemagglutinin response. In allogeneic MLR, SS non-T cells and macrophages stimulated normal allogeneic T cells less well than normal non-T cells and macrophages, suggesting a possible abnormality in the cells that stimulate in the allogeneic MLR.

INTRODUCTION

Sjögren's syndrome (SS)¹ is a chronic autoimmune disease characterized by lymphoid cell infiltration of exocrine glands, especially lacrimal and salivary glands (1). A combination of genetic, immunologic, and environmental factors may play a role in pathogenesis

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¹Abbreviations used in this paper: AMLR, autologous mixed lymphocyte reaction; Con A, concanavalin A; Δ cpm, counts per minute for cultures without mitogen; E-RFC, erythrocyte rosette-forming cells; FCS, fetal calf serum; M ϕ , macrophages; PHA, phytohemagglutinin; SLE, systemic lupus erythematosus; SS, Sjögren's Syndrome; TdR, thymidine.

(2). In SS, there is hypergammaglobulinemia, production of nonorgan-specific autoantibodies such as rheumatoid factors and antibodies to extractable nuclear antigens (3). By contrast, cellular immunity is decreased as shown by impaired delayed type skin reactions (4, 5) and decreased lymphocyte response to mitogens (4, 5). These findings suggest that immunoregulatory functions might be disturbed in SS.

The autologous mixed lymphocyte reaction (AMLR) measures the response of peripheral blood T cells to non-T cell surface membrane antigens which are closely linked to the HLA-DR locus (6). This reaction has been proposed as a mechanism by which T cells regulate lymphocyte function (7). The AMLR is decreased in systemic lupus erythematosus (SLE) (8, 9), and in several autoimmune-susceptible strains of mice such as NZB/NZW F₁ (B/W) and MRL/lpr.²

We studied the AMLR, allogeneic MLR, and mitogen responses in 25 patients with SS and in 18 normal controls. We found a decreased AMLR in SS, which may reflect an important immunoregulatory disturbance.

METHODS

Patient population. 25 patients seen in the Sjögren's Syndrome Clinic at the University of California, San Francisco, were studied. They were 3 males and 22 females, ranging from 20 to 81 yr of age. SS was diagnosed according to previous established criteria (1). The presence of keratoconjunctivitis sicca was based on positive staining with Rose-Bengal and decreased Schirmer test (10). Xerostomia was confirmed by labial salivary gland biopsy and parotid flow rate (11, 12). The diagnoses of rheumatoid arthritis and SLE were made by standard diagnostic criteria (13, 14). 15 SS patients had only glandular disease. Four patients had rheumatoid arthritis, two had SLE, two had pseudolymphoma, one had discoid lupus, and one had primary biliary cirrhosis with chronic thyroiditis and interstitial nephritis. With the exception of two patients with extra-

² Hom, J., and N. Talal. Decreased syngeneic mixed lymphocyte reaction in autoimmune-susceptible mice. Submitted for publication.

glandular disease who received 10 mg of prednisone, two patients with secondary SS who were treated with nonsteroidal anti-inflammatory drugs, and four patients with secondary SS who received 10 mg of prednisone, the patients did not receive any form of therapy. The normal group consisted of 18 age- and sex-matched individuals.

Separation of mononuclear cells. Separation of mononuclear cells was performed by the modified method of Bøyum et al. (15). Briefly, 35 ml of peripheral venous blood were drawn into a plastic syringe containing 25 U/ml of heparin (Liquaemin sodium, Organon Teknika Corp., Aurora, Col.). Heparinized blood was diluted with an equal volume of RPMI-1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) containing penicillin 100 U/ml, streptomycin 100 µg/ml, L-glutamine 2 mM, Hepes 10 mM (Sigma Chemical Co., St. Louis, Mo.) (designated as complete RPMI). 30–40 ml of the diluted blood were layered on 15 ml of Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, N. J.) in sterile 50-ml polypropylene tubes (Corning Glass Works, Scientific Products Div., Corning, N. Y.) and centrifuged at 400 g for 30 min at room temperature. The mononuclear cells collected from the interface were washed three times and resuspended in complete RPMI, supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories). The viability of cells was >95% by trypan blue exclusion. Portions of these cell suspensions were used for mitogen stimulation studies and enumeration of rosetted cells with sheep erythrocytes.

Purification of T cells, non-T cells, and macrophages. In preliminary experiments, cell purification and cultures were performed as described by Sakane et al. (8). Briefly, mononuclear cells were mixed with 2% suspension of neuraminidase (50 µg/ml, Sigma Chemical Co.)-treated sheep erythrocytes (Colorado Serum Co., Denver, Col.), for 1 h at 4°C and then centrifuged at 200 g for 5 min. The pellets were gently resuspended and the suspensions were layered over 15 ml of Ficoll-Hypaque as described above. Erythrocyte rosette-forming cells (E-RFC) collected from the bottom of the tubes were treated by 0.83% NH₄Cl buffered with trisaminomethane (Trizoma, Sigma Chemical Co.) to lyse sheep erythrocytes and washed three times. Non-RFC were recovered from the interface and washed three times. E-RFC and non-E-RFC are hereafter referred to as T and non-T cells. Both T and non-T cells were incubated overnight in complete RPMI supplemented with 10% heat-inactivated pooled human serum at 37°C in 5% CO₂. Non-T cells were depleted of adherent cells by removal of cells adhering to petri dishes and then treated with mitomycin-C (25 µg/ml, Sigma Chemical Co.) for 30 min. In 9 patients with SS and 12 normal controls, enrichment of adherent cells was performed. Briefly, unfractionated cells were adjusted to 2 to 3 × 10⁶/ml with complete RPMI with 20% FCS. Five ml of each suspension was incubated on coated petri dishes (1007, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) for 1 h at 37°C. Coated petri dishes were prepared beforehand by a 45-min incubation with 5 ml of poly-L-lysine (1 µg/ml, Sigma Chemical Co.) followed by an additional 45-min incubation with FCS. Nonadherent cells were collected from the plates by pipetting gently and then separated into T and non-T cells as described above. Adherent cells that remained on the plates were incubated with 5 ml of complete RPMI with 20% FCS, 3 mM ethylenediamine tetraacetic acid (EDTA, Sigma Chemical Co.) for 15 min at 4°C. The cells were removed from the plates by pipetting, followed by washing three times with complete RPMI and suspended in complete RPMI with 10% heat-inactivated pooled human serum. These cell suspensions were seeded into the wells of microtiter plates (Flow Laboratories, Rockville, Md.) as soon as possible to prevent further attachment of macro-

phages (Mφ) to the surface of test tubes. The preparation of adherent cells contained >85% of esterase positive cells, 5% of surface immunoglobulin-positive cells, and <5% of E-RFC. This fraction is referred to hereafter as Mφ for convenience. In both methods, preparation of T cells contained >85% of E-RFC, 1% of surface immunoglobulin-positive cells, and <1% of esterase-positive cells; non-T cells contained 70% of surface immunoglobulin-positive cells, 25% of esterase positive cells, and <5% of E-RFC.

Mitogen stimulation. Unfractionated cells (2 × 10⁵) were incubated with 1 µg of purified phytohemagglutinin (PHA) (Burroughs-Wellcome & Co., Reagent Div., Greenville, N. C.), 20 µg of concanavalin-A (Con-A) (Miles Laboratories, Elkhart, Ind.), or 20 µl (1:10 dilution) of pokeweed mitogen (Gibco Laboratories) in 200 µl of complete RPMI with 20% FCS for 72 h at 37°C in 5% CO₂. These doses of mitogen were shown to be optimal for maximum stimulation in preliminary studies. All cultures were performed in triplicate. Tritiated thymidine [*methyl*-³H]TdR, specific activity in 6.0 Ci/mmol, (Schwarz/Mann, Div. of Becton Dickinson & Co., Orangeburg, N. Y.) was added 20 h before harvesting with a dose of 1 µCi/well. Cultures were harvested on filter papers (Whatman, Inc., Clifton, N. J.) by semiautomatic cell harvester (Otto-Hillier Co., Madison, Wisc.). The radioactivity was determined in a liquid scintillation counter (model 3380, Packard Instrument Co., Downers Grove, Ill.). The data are expressed as the difference in counts per minute for cultures containing mitogen and counts per minute for cultures without mitogen (Δcpm).

Mixed lymphocyte cultures. Cultures were set up in triplicate and consisted of 200 µl complete RPMI with 10% pooled human serum containing 1 × 10⁵ responding T cells and 1 × 10⁵ stimulating MMC-treated non-T cells or 5 × 10⁴ Mφ. Maximum proliferation in allogeneic MLR and AMLR between T and non-T cells occurred on day 6 or 7, and in AMLR between T and Mφ, on day 7 or 8. All cultures were incubated for 144 h at 37°C in 5% CO₂. Pulsing and harvesting were performed as described above. The data are expressed as the difference between counts per minute from complete cultures (containing responding T cells and stimulating non-T cells or Mφ, either autologous or allogeneic) and the sum of counts per minute from cultures containing T cells alone and stimulating cells alone.

Detection of antilymphocyte antibody. The modified method of Terasaki was used (16). Briefly, 50 µl of 4 × 10⁶ ml of mononuclear cells were incubated with 50 µl of serum to be tested at 15°C for 30 min followed by 50 µl of rabbit complement (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) at 15°C for 180 min. The viability of cells was measured by trypan blue exclusion. Serum that killed 20% or more cells was considered positive for cytotoxicity.

Data analysis. Data were analyzed on a computer (Wang Laboratories, Inc., Lowell, Mass.), programmed to reject individual data points more than three standard deviations from the calculated means. Wilcoxon rank-sum test was used for statistical methods.

For the first 16 SS patients and 6 normal controls, the AMLR was performed by the method described by Sakane et al. (8). For the next 9 SS patients and 12 normal controls, we used the other method described above to recover adherent cells. Because both methods gave similar responses in AMLR and allogeneic MLR, all data were analyzed together.

RESULTS

The AMLR between T cells and non-T cells in patients with SS and in normal controls. The AMLR between T cells and non-T cells were studied in 25 pa-

TABLE I
Correlation between the Response in AMLR and Parameters in SS

AMLR	Age	Focus score	Serum Ig	Antilymphocyte antibody	PHA response	Con-A response
			mg/dl	% positive	(Net incorporated [³ H]TdR)	(Net incorporated [³ H]TdR)
Normal (n = 10)	54±16	4.5±3.5	1,473±639	20%	39,010±39,333	50,134±22,859
Abnormal (n = 15)	57±16	4.4±3.1	1,758±614	20%	35,424±26,721	33,096±23,946*

* P < 0.001.

tion between SS T and SS allogeneic non-T cells revealed the lowest response, probably related to shared histocompatibility antigens.

The AMLR between T cells and Mφ in SS and normal controls. The AMLR between T cells and purified Mφ was performed in 9 SS patients and in 12 normal controls (Fig. 4). The AMLR in the SS patients was lower when compared with the normal controls, (P < 0.02).

The allogeneic MLR between T cells and Mφ in SS patients and in normal controls. There is no difference in the response of normal and SS T cells to stimulation by normal allogeneic Mφ (Table III). However, SS Mφ were unable to stimulate normal allogeneic T cells as well as normal Mφ (P < 0.001).

DISCUSSION

The AMLR is a phenomenon by which T cells proliferate in response to non-T cell surface antigens (7, 8). This reaction has two important characteristics of an immune response, immunological memory and specificity (18). The biological significance of this reaction is unclear, although it may represent a mechanism by which T cells regulate lymphocyte function (7).

In our experiments, 15 of 25 SS patients (60%) had decreased AMLR, including 8 with only glandular disease who were not on systemic medications. The decreased AMLR did not correlate with age, salivary

gland focus score, serum immunoglobulin concentration, the titer of antilymphocyte antibody, or PHA response. However, the AMLR in SS was decreased in patients who also had a decreased response to Con-A.

Two patients with pseudolymphoma showed absent AMLR response. Pseudolymphoma is a transitional stage to malignant lymphoproliferation that occurs in SS patients. This observation raises a possible relationship of the AMLR to immune surveillance.

The AMLR is decreased in SLE (8, 9), chronic lymphocytic leukemia (19) and infectious mononucleosis (19). The analogous interaction between syngeneic spleen T and non-T cells is markedly diminished in New Zealand black mice (20), an experimental model

TABLE II
Allogeneic MLR between T Cells and Non-T Cells from Normal Controls and Patients with SS

Responding T cells	Stimulating non-T cells	Mean of Δcpm
SS	Normal	55.224*
SS	SS	24.461*
Normal	SS	33.281*
Normal	Normal	63.184

* Significantly different from response to normal non-T cells (P < 0.001).

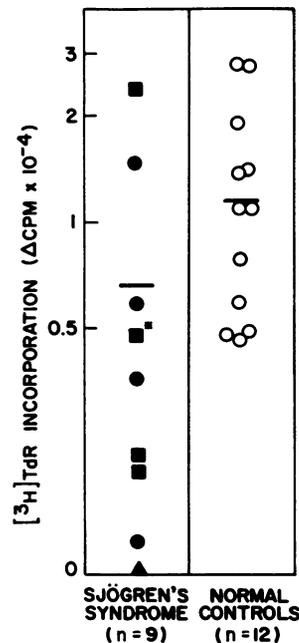


FIGURE 4 The AMLR between T cells and Mφ in SS and normal controls. The patients with SS showed decreased response. The data points derived from patients treated with prednisolone are starred. ●, glandular disease only; ▲, pseudolymphoma; ■, associated connective tissue disease.

TABLE III
Allogeneic MLR between T Cells and M ϕ from Normal Controls and Patients with SS

Responding T cells	Stimulating M ϕ	Mean of Δ cpm
SS	Normal	48.771
SS	SS	ND
Normal	SS	35.431
Normal	Normal	54.502

* Significantly different from response to normal M ϕ ($P < 0.001$).

for human lupus. It is also decreased in several other autoimmune susceptible strains such as NZB/NZW F₁ (B/W) mice, BXSB/mp, and MRL/lpr mice.² Sakane et al. (8, 21), found that the decreased response in active lupus was due both to defective responding T cells and to defective stimulating cells. However, Kuntz et al. (9) concluded that a B cell defect alone might cause the reduced response in SLE. In chronic lymphocytic leukemia and infectious mononucleosis, this defect mainly exists in B cells (18, 19).

There is considerable evidence that the responding cells in AMLR are T cells (22, 23). The nature of the stimulating cells is controversial. Opeltz (24) found that a B cell-rich population purified by goat-anti-human immunoglobulin column fractionation was the main stimulator. However, Kuntz et al. (7) attributed the stimulating activity to non-T cells that had characteristics of K lymphocytes. Removal of phagocytic or adherent cells in the B cell population increased its capacity to stimulate autologous T cell proliferation (7). Another study demonstrated that B cells were the major stimulators and that monocytes obtained from velocity centrifugation failed to stimulate autologous T cells (23). Katz and Fauci (24) showed that removal of adherent cells from non-T cell populations by Sephadex G-10 columns augmented the AMLR. Beale et al. (25) reported that monocytes produced the highest response, null cells an intermediate response, and B cells, a negligible level of stimulation in this response. Stobo et al.³ found that both B cells and M ϕ can stimulate. In our experiments using M ϕ , there were vigorous responses in both allogeneic MLR and AMLR in normal individuals. These different results may be explained by different techniques of cell purification and identification. The purity of M ϕ that we prepared was >85% as identified by esterase staining. We found that 15% of non-T cells (B plus null cells) contaminating this preparation could not stimulate autologous T cells, and that greater numbers of M ϕ could inhibit AMLR

³ Personal communication.

(data not shown). This may partly explain the failure of M ϕ to stimulate in some studies.

There may be several explanations for the decreased AMLR in SS. This can include a responding T cell defect, a stimulating non-T cell defect, or abnormalities in T and non-T cell interactions. T cell function in SS is abnormal as shown by decreased mitogen response and diminished delayed type skin reactivity (4, 5). Antilymphocyte antibody could be cytotoxic to autoreactive T cells but we found no correlation between decreased AMLR and the titer of antilymphocyte antibody. The association of a decreased AMLR with decreased Con-A response suggests that the cell population activated by Con-A may belong to the same population as the AMLR responsive cell. Con-A induces T suppressor cell activity (26). A correlation between AMLR and suppressor activity was demonstrated in SLE by Sakane et al. (8). In addition, suppressor cells that inhibit allogeneic MLR and antibody production by B cells are generated during the AMLR (27, 28). These findings support the idea that autologous reactive T cells are quantitatively or qualitatively defective. However, a non-T cell defect may also be present. The observation that SS B cells and M ϕ had reduced stimulating ability in allogeneic MLR suggests possible abnormalities in non-T cell populations. It should be understood that these may not be the same cells that stimulate in AMLR. Immune complexes that bind Fc receptors may inhibit the stimulatory capacity of non-T cells. Other serum factors may inhibit the expression of Ia antigens on the surface of non-T cells causing decreased AMLR. However, overnight incubation of non-T cells, which allows shedding of such factors, did not restore stimulating ability. Intrinsic defects of non-T cells are another possibility. Abnormalities in the metabolism of non-T cells may cause disturbed stimulatory capacity or may give diminished viability during culture (although cell viability after the termination of culture showed no difference between SS and normal controls).

The AMLR may represent internal communication between T cells and non-T cells. Decreased communication or interaction of these cells may induce aberrations of immunoregulation leading to autoimmune diseases (29). These results suggest that the decreased AMLR may reflect an immunoregulatory imbalance in SS.

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