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Impaired Lymphocyte Transformation and Delayed Hypersensitivity in Sjögren's Syndrome *

BRIGID G. LEVENTHAL,† DONALD S. WALDORF,‡ AND NORMAN TALAL

(From the National Cancer Institute and National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.)

Summary. ***In vitro*** **lymphocyte transformation in response to phytohemagglutinin and streptolysin O and** ***in vivo*** **skin sensitization to 2,4-dinitrochlorobenzene has been studied in patients with Sjögren's syndrome and in normal controls of comparable age and sex. Both the** ***in vivo*** **and** ***in vitro*** **responses were significantly impaired in the Sjögren's patients as compared to the controls. This lack of response to mitogenic agents is probably due to an intrinsic defect in the lymphocytes rather than to a serum factor. The abnormalities were less marked in patients whose disease was localized to the parotid and lacrimal glands than in those with generalized disease, i.e., with complicating rheumatoid arthritis or pseudolymphoma.**

Introduction

Sjögren's syndrome is a chronic disorder characterized by dry mouth and dry eyes (the sicca syndrome) due to decreased salivation and lacrimation (1). The salivary and lacrimal glands may show lymphocytic infiltration and atrophy of acini. Over half the patients have rheumatoid arthritis. Rheumatoid factor and several other "autoantibodies" are commonly found in the serum (2). Talal and Bunim reported the development of reticulum cell sarcoma in three patients with Sjögren's syndrome (3). Other examples of this association have been observed (4–6). More recently, Talal, Sokoloff, and Barth (7) have described patients with Sjögren's syndrome and "pseudolymphoma," a condition characterized by lymphadenopathy, splenomegaly, purpura, lymphoplasmacytic pulmonary infiltrates, and macroglobulinemia. The involved tissues have architectural features suggestive but not diagnostic of a malignant lymphoma.

Peripheral blood lymphocytes from normal individuals can be stimulated to transform into large "blast" cells *in vitro* by nonspecific mitogens such as phytohemagglutinin (PHA) (8, 9). Specific antigens will also induce transformation in lymphocytes from sensitized individuals (10, 11). These responses are impaired in patients with lymphomatous disorders such as chronic lymphatic leukemia (12–14) and Hodgkin's disease (15, 16). Because Sjögren's syndrome may in some patients be associated with the development of lymphoma, it seemed of interest to investigate the peripheral lymphocytes from patients with this disorder. The following studies were therefore undertaken to determine *1*) the ability of PHA and streptolysin O (SLO) to stimulate *in vitro* transformation and *2*) the capacity of the patients to develop delayed hypersensitivity to a specific contact allergen, 2,4-dinitrochlorobenzene (DNCB). Lymphocytes from the majority of patients with Sjögren's syndrome were found to be functionally abnormal in these studies and resembled lymphocytes found in Hodgkin's disease or chronic lymphatic leukemia.

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† Address requests for reprints to Dr. Brigid G. Leventhal, Leukemia Service, Medicine Branch, National Cancer Institute, Bethesda, Md. 20014.

‡ Current address: Dept. of Dermatology, New York University School of Medicine, New York, N. Y.

Methods

Thirty-three patients with manifestations of Sjögren's syndrome for 1–21 years were studied. All were patients at the Clinical Center of the National Institutes of Health. They ranged in age from 30–73 years (mean 55). Thirty-one were female, and two were male. The diagnosis of Sjögren's syndrome required the presence of at least two of the following: keratoconjunctivitis sicca, chronic or recurrent salivary gland enlargement, rheumatoid arthritis, decreased salivary flow, or a characteristically abnormal secretory sialogram. The parotid gland was biopsied in 11 patients and showed the typical "benign lympho-epithelial lesion" (17).

The patients were divided into three groups on the basis of the following clinical findings: *a*) sicca syndrome alone (13 patients), *b*) sicca syndrome plus rheumatoid arthritis (12 patients), and *c*) sicca syndrome plus lymphoma (1 patient) or pseudolymphoma (7 patients). This group included the 2 males. The patient with lymphoma is a 5-year survivor after radical surgery and radiotherapy for reticulum cell sarcoma of the inguinal lymph nodes (4). Seventeen patients with classical rheumatoid arthritis (13 females and 4 males) ranging in age from 2–60 years were also studied. Because lymphocyte responsiveness may decrease with age (18), an attempt was made to match the controls with the patients. The normal controls were 20 blood bank donors (18 females, 2 males) ranging in age from 25–68 years (mean 50). They had no known disease and were not taking medication.

Lymphocyte culture technique. Thirty to 50 ml of venous blood was drawn through a 19-gauge needle into glass syringes containing 300–500 U of heparin. The blood was allowed to sediment in a glass tube for 90 minutes at 37° C after which the leukocyte-rich plasma was drawn off gently into another syringe. Total and differential leukocyte counts were performed. One ml of leukocyte-rich plasma was added to each 10-ml glass culture bottle. Two ml of Eagle's minimal essential medium supplemented with glutamine (40 mmoles per 100 ml),[1] penicillin, and streptomycin (1,000 U per 100 ml) [1] was then added to each culture. The final culture volume was 3 ml. Phytohemagglutinin M (0.05 ml) [2] or streptolysin O (0.1 ml) [2] was added to the appropriate bottles, and the cultures were incubated for 5 days at 37° C. Sterile conditions were maintained and duplicate cultures were set up for each measurement. The mean number of lymphocytes added per milliliter culture fluid was 800,000 (range 0.3 to 1.8×10^6).

Morphologic examination. After 5 days of incubation, cultures to be examined microscopically were centrifuged at 300 g for 7 minutes. The cell button was resuspended in 0.1 ml of calf serum and spread on glass slides. Smears were stained with either Wright's stain or Giemsa stain after fixation in 95% ethanol for 10 minutes. A minimum of 300 leukocytes was counted on each slide, and the entire slide was traversed at least once so as to include the large cells at the edge of the smear. A cell was considered transformed if it had a nuclear diameter at least twice that of an adjacent red cell with finely dispersed nuclear chromatin and cytoplasmic basophilia. The majority of these cells also contained nucleoli. Macrophages were distinguished by their gray-pink cytoplasm and greater cytoplasmic:nuclear ratio relative to that of the transformed lymphocyte. Any cell with nuclear pyknosis or karyorrhexis was considered nonviable. Approximately 30% of the cells in unstimulated cultures from both patients and controls were in this category. The number of transformed lymphocytes was expressed as a percentage of total leukocytes counted, including those considered nonviable. The counts were reproducible with a mean variation on simultaneous duplicate PHA cultures of 8% for the controls and 20% for the patients.

Ten of the patients were studied on two or more separate occasions within a 6-month period and showed a consistent response of either normal or impaired transformation.

Resuspension experiments. Lymphocytes from patients and controls were centrifuged at 300 *g* for 7 minutes, washed once with Eagle's minimal essential medium, and then resuspended in medium. The cells were added to culture bottles in quantities that would produce final cell concentrations of 1×10^6 cells per ml. Medium was added to a volume of 2 ml, after which 1 ml of the cell-free serum or plasma to be studied was added. These cultures were harvested as described above.

Incorporation of thymidine-^3H. Thymidine-^{3}H incorporation was studied as a measure of DNA synthesis in duplicate cultures from 10 patients and 6 controls. Six μc of thymidine-^{3}H (6.7 c per mmole) [3] was added to each culture bottle 2 hours before harvesting. At the end of the incubation period, cultures were centrifuged for 5 minutes at 1,000 *g* and the cell button was resuspended and washed three times with 5-ml aliquots of 0.85% NaCl (buffered at pH 7.4) and twice with 5 ml of 95% ethanol. The button was dissolved in 1 ml of hydroxide of hyamine [4] by heating at 60° C for 15 minutes. Five ml of 0.6% 2,5-diphenyloxazole [3] in toluene was added to each solution, which was then transferred to a glass counting bottle. The tubes were rinsed with a second 5 ml, which was also added to the counting bottles. The specimens were counted in a Packard Tri-Carb liquid scintillation counter (model 3003). The efficiency for ^{3}H was 48%. Appropriate corrections for quenching were made.

Zero time controls were performed by adding 5 μc of thymidine-^{3}H to unstimulated cultures and centrifuging immediately. These contained 100 to 200 cpm per culture. Cells that have been exposed to PHA for 5 days are largely found in clumps, and it is therefore not possible to do an accurate cell count on the day the cultures are

[1] Flow Labs., Rockville, Md.

[2] Difco Labs., Detroit, Mich.

[3] New England Nuclear Corp., Boston, Mass.

[4] Packard Instrument Company, Inc., Downers Grove, Ill.

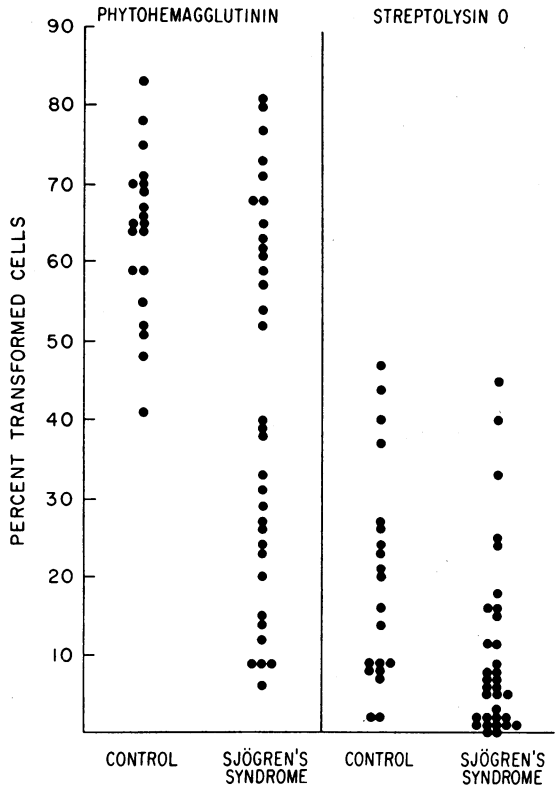

FIG. 1. PER CENT TRANSFORMED CELLS AFTER IN VITRO STIMULATION. Transformed lymphocytes in response to both phytohemagglutinin and streptolysin O in 20 controls is compared with that of all 33 patients with Sjögren's syndrome.

harvested. For this reason data are expressed in terms of number of lymphocytes added on day 1.

Delayed hypersensitivity to DNCB. Sensitizing doses of DNCB were applied to 17 normal subjects (aged 59 to 69 years), 23 patients with Sjögren's syndrome, and 15 patients with rheumatoid arthritis. Concurrent lymphocyte transformation studies were performed on the patients with Sjögren's syndrome, but were not routinely performed on the other subjects. The sensitizing regimen consisted of the application of 2,000 μg of DNCB in 0.1 ml acetone to an area of upper arm skin circumscribed by a 2-cm diameter plastic ring. The solution was allowed to evaporate, and the area was covered by a small bandage for 5 to 7 days. To test for previous sensitization to the chemical, doses of 100 μg and 50 μg DNCB in 0.1 ml acetone were applied in similar manner to the forearm. These sites were covered for 48 hours and observed for signs of delayed hypersensitivity (erythema, edema, vesiculation). Challenge doses of 100 μg and 50 μg DNCB were applied to the skin of the opposite forearm at 14 days to determine whether sensitization had developed.

Statistical methods. Morphologic and radioisotopic data were compared by the rank sum test of Wilcoxen (19). Skin sensitization results were evaluated by the chi square test (20).

Results

The percentage of lymphocytes transformed after PHA stimulation in cultures from the 20 control subjects and the 33 patients with Sjögren's syndrome is shown in Figure 1. The control values range from 41 to 83% (median 65%); the patients' values range from 6 to 81% (median 39%). These numbers differ significantly ($p = 0.001$). The great variability in the patient group apparently reflects a bimodal distribution into normal and poor (40% or less transformed cells). Eighteen of the 33 patients showed a poor response.

The responses to SLO showed a similar pattern. The control values ranged from 2 to 47% (median 16%), the patients' from 0 to 45% (median 6%). These values differ from one another with $p = 0.002$. A poor response to SLO is arbitrarily defined as one of less than 6%. Two

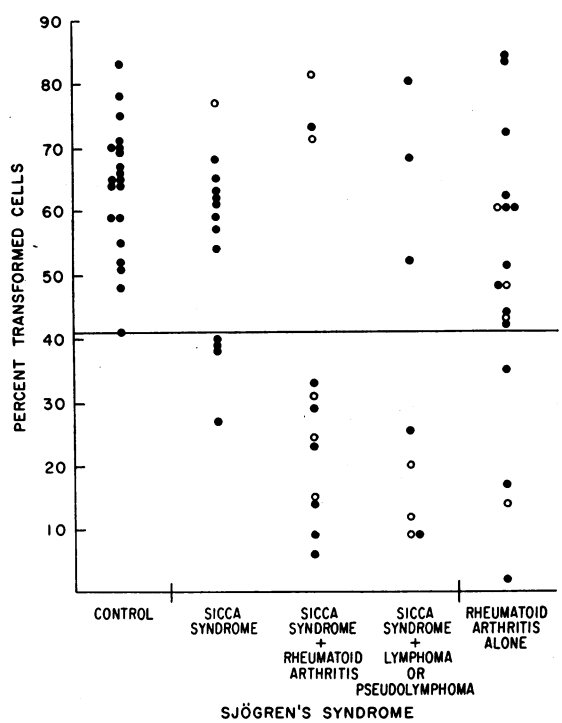

FIG. 2. RESPONSE TO PHYTOHEMAGGLUTININ. The patients with Sjögren's syndrome are divided into clinical subgroups and compared with controls and with patients who have uncomplicated rheumatoid arthritis. Open circles represent patients receiving corticosteroids.

of the control subjects and 17 of the 33 patients showed such a response.

In Figure 2 the patients are divided into clinical subgroups and their response to PHA tabulated. Transformation is impaired in 4 of 13 (33%) patients with sicca syndrome, 9 of 12 (75%) with sicca syndrome plus rheumatoid arthritis, and 5 of 8 (60%) with sicca syndrome plus lymphoma or pseudolymphoma. Results of studies in the 17 patients with rheumatoid arthritis are also shown. They range from 2 to 84% (median 48%), and 4 of the 17 (24%) show a poor response. The response of the group resembles that of the patients with sicca syndrome alone rather than the more marked abnormality seen in the other two groups.

The responses to SLO are presented in Figure 3. Again, the patients with sicca syndrome complicated by rheumatoid arthritis or pseudolymphoma are more abnormal than those with sicca syndrome alone. The patients with rheumatoid arthritis have values from 1 to 44% (median 9%). The results of both experiments are summarized in Table I. They suggest a more severe lymphocyte derangement in the groups with sicca syndrome complicated by rheumatoid arthritis or pseudolymphoma.

Incorporation of thymidine-^3H. In the 6 control subjects there is a general correlation between the number of lymphocytes added to each culture and the amount of isotope incorporated (Table II). Expressed as counts per minute per 10^6 lymphocytes added, the range is from 31,442 to 88,000 with a median of 69,500. This range is similar in magnitude to the variation from 40 to 80% in transformed cells found in normal subjects on morphologic study. In the 10 patients with Sjögren's syndrome there is no correlation between the number of lymphocytes added and the amount of isotope incorporated, again emphasizing the fact that some responded to PHA and some did not.

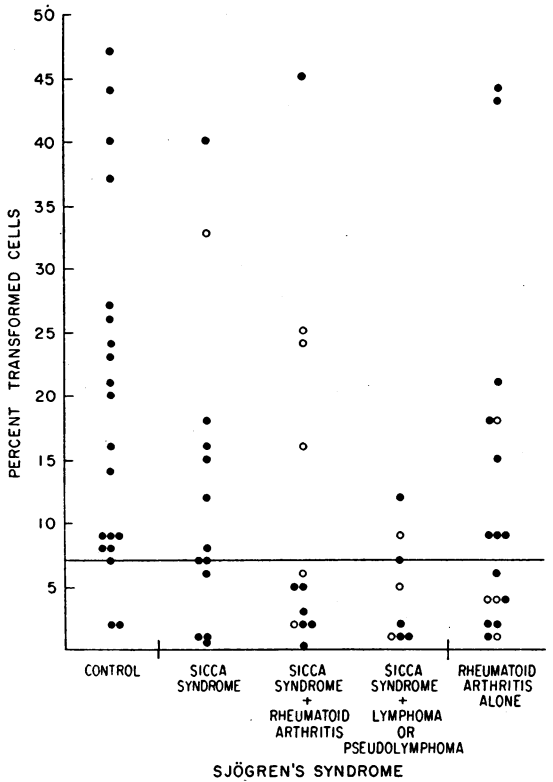

FIG. 3. RESPONSE TO STREPTOLYSIN O. Patients are divided into clinical subgroups. Open circles represent patients receiving corticosteroids.

TABLE I

Lymphocyte transformation

	Total patients	Number of patients with response			
		Normal to PHA and SLO*	Poor to PHA only	Poor to SLO only	Poor to PHA and SLO
Sicca syndrome	13	6	3	3	1
Sicca syndrome plus rheumatoid arthritis	12	2	2	1	7
Sicca syndrome plus lymphoma or pseudolymphoma	8	2	1	1	4
Sjögren's syndrome (TOTAL)	33	10	6	5	12
Rheumatoid arthritis	17	9	0	4	4

* PHA = phytohemagglutinin; SLO = streptolysin O.

TABLE II

Incorporation of thymidine-^3H after PHA stimulation

	LA × 10^6/ml*	*cpm*	*cpm*/10^6 *lymphocytes*
Control subjects			
1	0.78	24,525	31,442
2	0.84	33,000	39,286
3	0.94	81,000	86,170
4	1.0	88,000	88,000
5	1.3	92,000	70,769
6	1.6	108,520	67,816
Median	0.96	84,000	69,500
Patients with Sjögren's syndrome			
1	0.32	7,500	23,438
2	0.33	12,500	37,879
3	0.41	5,000	12,295
4	0.58	100,000	172,414
5	0.74	45,000	50,811
6	0.78	1,625	2,083
7	0.80	2,200	2,750
8	0.81	393	485
9	1.10	28,000	25,454
10	1.83	12,000	6,557
Median	0.76	9,700	17,500

* LA = lymphocytes added.

The range for counts per minute per 10^6 lymphocytes is from 485 to 172,414 with a median of 17,500. The normal subjects and the patients are significantly different from one another (p less than 0.01).

Resuspension experiments. The impaired transformation observed in these patients could be attributed either to a plasma factor or to some defect in the cells themselves. To distinguish between these two possibilities, the experiments shown in Table III were performed. Lymphocytes from four patients were studied in the patients' own plasma (autologous) and after resuspension in calf serum. One patient's cells (L.B.R.) were resuspended in control human plasma. No significant increase in response was noted after resuspension. In two other experiments, control cells were studied in autologous plasma and in plasma from patients D.K. and I.L., whose sera contained rheumatoid factor in titers of $> 1:8,000$ and $1:256$, respectively. The control cells transformed normally in the patients' plasma. This series of experiments suggest that the depressed transformation observed in Sjögren's syndrome is not due to an inhibitory factor, such as the rheumatoid factor, present in the patient's plasma. Since transformation was still decreased after resuspension of patient's cells in control plasma or calf serum, it

TABLE III

Resuspension of lymphocytes in serum or plasma: per cent transformation after PHA

	Plasma or serum source		
Cell donor	Autologous	Homologous	Calf
E.S.	14		11
D.K.	12		24
M.S.	15		28
I.L.	34		29
L.B.R.	12	24	
Control	69	91 (D.K.)	
Control	60	54 (I.L.)	62

TABLE IV

Transformation in patients on steroids

				Per cent transformation	
Patient	Steroid	Dosage	Duration	PHA	SLO
Sjögren's syndrome		*mg*	*years*		
M.H.	β-Methasone	0.9	13	81	16
	(prednisone equivalent)	7.5			
S.J.	Prednisone	5	2	77	33
L.J.	Prednisone	7.5	12	71	25
V.M.	Prednisone	12.5	10	24	5
E.O.	Prednisone	7.5	11	23	5
I.W.	Prednisone	15	$\frac{3}{4}$	20	5
M.S.	Prednisone	10	$3\frac{1}{2}$	15	23
L.B.R.	Prednisone	15	$1\frac{1}{4}$	12	0.5
H.H.	Prednisone	10	15	9	9
Rheumatoid arthritis					
M.T.	Prednisone	10	3	60	1
E.A.	Prednisone	5	5	48	4
M.G.	Prednisone	12.5	5	43	18
C.S.	Prednisone	12.5	13	14	4

TABLE V

Delayed hypersensitivity to 2,4-dinitrochlorobenzene (DNCB)

Subjects	Number studied	Sensitized to DNCB	% Positive	Significance compared to normals
Normal	17	16	94	
Sjögren's syndrome*				
SS	9	5	55	
SS + RA	9	1	11	
SS + PL	5	2	40	
Total	23	8	35	$p < 0.001$
Rheumatoid arthritis	15	10	67	$p > 0.05$

* SS = sicca syndrome, RA = rheumatoid arthritis, PL = pseudolymphoma.

seems likely that a defect in the lymphocytes themselves is responsible for the abnormal response to mitogens.

Corticosteroids. Corticosteroids have been shown to depress transformation *in vitro* (21). Nine of these 33 patients with Sjögren's syndrome and 4 of 17 with rheumatoid arthritis were receiving corticosteroid therapy at the time of study. The medication, dosage, and duration of treatment are shown in Table IV. These patients' values are shown by open circles in Figures 2 and 3. Removing them from consideration does not markedly alter values obtained for the group as a whole. Thus, although corticosteroids may contribute to the depressed transformation response in some patients, it is not the primary cause of hyporesponsiveness in the group. Two patients on steroids (M.S. and L.B.R.) still showed poor transformation after the cells were washed and resuspended in serum or plasma (see Table III). There was no correlation between lymphocyte transformation and the administration of salicylates.

Delayed hypersensitivity to DNCB. None of the subjects tested demonstrated previous sensitization to DNCB. Sixteen of the 17 normal subjects (94%) were made reactive to the agent, a finding consistent with previously reported results (22). Of the 23 patients with Sjögren's syndrome, only 8 could be sensitized to DNCB, a degree of responsiveness significantly below that of the normals (p less than 0.001), Table V). Five of nine (55%) patients with sicca syndrome alone were sensitized, whereas only 1 of 9 (11%) with sicca syndrome and rheumatoid arthritis and two of five (40%) with pseudolymphoma responded to the chemical. Ten of 15 patients with rheumatoid arthritis (67%) were sensitized to DNCB. This incidence of responsiveness was intermediate between the normal subjects and Sjögren's patients and not significantly different from either group.

TABLE VI

Comparison of in vivo and in vitro responses in patients with Sjögren's syndrome

In vitro response abnormal to:	DNCB negative	DNCB positive
Both PHA and SLO	6	2
PHA only	4	0
SLO only	1	2
Normal response to PHA & SLO	4	3
Total	15	7

Comparison of lymphocyte transformation and DNCB sensitization. Lymphocyte transformation was studied in 22 of 23 patients in whom DNCB sensitization was attempted (Table VI). There was no apparent correlation between the *in vitro* response to PHA and SLO and the *in vivo* response to DNCB in individual patients. This suggests that the two tests may be measuring different parameters of lymphocyte function.

Discussion

This report demonstrates that the circulating lymphocytes in a majority of patients with Sjögren's syndrome have a decreased capacity to respond when stimulated with mitogens *in vitro* or or with DNCB *in vivo*. This impairment is similar to the lymphocyte abnormalities and skin anergy reported in chronic lymphatic leukemia (12–14), Hodgkin's disease (15, 16, 23) sarcoidosis (24, 25), and ataxia telangiectasia (26, 28). The

in vitro hyporesponsiveness appears to be an intrinsic cellular abnormality since transformation did not improve when the patients' washed lymphocytes were resuspended in calf serum or control human plasma.

The functional abnormality of peripheral blood lymphocytes may be part of a more widespread derangement of lymphocytes and related cells. The most characteristic feature of Sjögren's syndrome is a diffuse lymphocytic infiltration of the salivary and lacrimal glands. In addition, certain patients have developed extraglandular lymphoid infiltrates with features of lymphoma or pseudolymphoma. The lymphomas have involved the lymph nodes, the liver, and the gastrointestinal tract. The pleomorphic pseudolymphomatous infiltrates have involved the lymph nodes and lung. As reported here 50% of patients with lymphoma or pseudolymphoma have functionally abnormal peripheral blood lymphocytes. This defect of circulating cells is not restricted to these patients, however. It is present in approximately half the subjects studied, and is particularly striking when the sicca syndrome and rheumatoid arthritis are present together.

Patients with the sicca syndrome and rheumatoid arthritis have a lesser degree of salivary dysfunction and fewer serologic abnormalities than patients with the sicca syndrome alone (2, 4). In the NIH series precipitating, antinuclear, and complement-fixing antibodies were more varied and four to eight times more frequent in patients with the sicca syndrome alone than in patients with sicca syndrome and rheumatoid arthritis (2). Moreover, pseudolymphoma tends to develop in patients with the sicca syndrome alone (4). It is remarkable, therefore, that patients with sicca syndrome and rheumatoid arthritis should have a greater degree of circulating lymphocyte dysfunction. This cannot be attributed to the synovial disease *per se,* since patients with rheumatoid arthritis alone do not demonstrate this degree of lymphocyte impairment.

The lymphocytes infiltrating the salivary and lacrimal glands in Sjögren's syndrome may originate *in situ* from intraglandular lymphoid structures. The intraparotid lymph nodes are known to undergo hyperplasia in this disease (29). When the lymphoid infiltrates are confined to the salivary and lacrimal glands, peripheral blood lymphocyte function is least impaired. When the disease is widely distributed as in patients who have accompanying pseudolymphoma or rheumatoid arthritis, the greatest degree of impairment is found.

Good, Peterson, Finstad, and Gabrielsen (30) have drawn a distinction between peripheral lymphoid organs (such as spleen and lymph nodes) and central lymphoid tissues (such as thymus and bursa of Fabricius in fowl). Lymphocytes are thought to arise in the latter and travel to the periphery where they are presumed to acquire new functional characteristics. Central lymphoid organs have the following characteristics: *1*) origin from epithelial structures, *2*) association with digestive organs, *3*) association of abnormalities with the development of malignant lymphomas (e.g., in ataxia telangiectasia or congenital sex-linked agammaglobulinemia). The intraparotid lymphoid structures in patients with Sjögren's syndrome appear to fulfill all of the above requirements for central lymphoid organs (4).

Another distinction between central and peripheral lymphocytes may be the ability to respond to mitogen stimulation *in vitro.* Lymphocytes from peripheral lymph nodes and spleen respond to PHA in the same manner as peripheral blood lymphocytes (31, 32). By contrast, thymic lymphocytes transform poorly when exposed to PHA *in vitro* (31, 33). This may be a general property of all central lymphocytes. We hypothesize that the presumably central lymphocytes infiltrating the parotid gland in patients with Sjögren's syndrome might also appear in the circulation (4). These lymphocytes, because of their central lymphoid properties, would be relatively unresponsive to mitogens and unable to participate effectively in delayed hypersensitivity reactions, thus accounting for the results observed in this study. Our data are in accord with the concept that varying populations of lymphocytes with different tissue origins and different functional properties may be present in the peripheral blood at the same time.

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