

# Analyses of Lymphocytes from Patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus

## OCCURRENCE OF INTERFERING COLD-REACTIVE ANTILYMPHOCYTE ANTIBODIES

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**ABSTRACT** Large percentages of the lymphocytes from some patients with rheumatoid arthritis and systemic lupus erythematosus were densely covered with Ig demonstrable by immunofluorescence, which was occasionally present in the form of caps. The amount and character of the Ig staining depended largely on the procedures used in the isolation and washing of the lymphocytes. Cold-reactive antilymphocyte antibodies present in many sera were primarily responsible for these variations. Overnight culture of the lymphocytes proved to be an efficient procedure for the removal of adsorbed antibody. Some evidence was also obtained for the presence of circulating immune complexes and exogenous rheumatoid factor molecules on the lymphocyte surface. Thus on freshly isolated cells the demonstration of surface Ig proved to be an unreliable marker of bone marrow-derived (B) cells in these disease; the actual percent of B cells with intrinsic surface Ig was often markedly decreased. In patients with systemic lupus erythematosus, this reduction was in agreement with the low numbers of cells that had a receptor for aggregated IgG. The mean percentage of thymus-derived (T) cells in both diseases was slightly greater than the normal level.

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The concentrations of lymphocytes in joint fluids from patients with rheumatoid arthritis were often greater than levels found in blood. T cells primarily accounted for this increase. The T cells typically formed unusually dense rosettes with sheep erythrocytes. B lymphocytes were proportionally much diminished. Evidence was obtained for the existence of a major joint fluid lymphocyte population that lacked all assayed surface markers.

## INTRODUCTION

Considerable evidence has accumulated for a variety of immunological alterations in patients with rheumatoid arthritis (RA)<sup>1</sup> and systemic lupus erythematosus (SLE), although the basic etiologies remain unknown. Recent methodologic advances in the study of lymphocytes have furthered the understanding of a number of disorders, particularly the immunodeficiency and lymphoproliferative groups (1, 2). Utilizing similar methods, several studies have been made of patients with RA and SLE (3-9). Although the data of these investigators differed regarding the composition and character of the peripheral blood lymphocyte populations, the predominant conclusion was that the surface Ig-bearing lymphocytes were increased, implying an increase in the bone marrow-derived (B) cell population.

However, there are several factors present in these diseases that may complicate the interpretation of cell

<sup>1</sup>Abbreviations used in this paper: B, bursal-equivalent or bone marrow-derived (lymphocytes); FCS, fetal calf serum; HBSS, Hanks' balanced salt solution (Ca-Mg<sup>2+</sup>, lacking calcium and magnesium); PBS-BSA, pH 7.4 phosphate-buffered saline, 2% bovine serum albumin, 0.02% sodium azide; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T, thymus-derived (lymphocytes).

surface markers by interfering with the assay procedures. Antibodies to endogenous antigens, particularly those to the lymphocyte surface, and circulating immune complexes occur that may be demonstrated by direct cytotoxicity, inhibition of mixed lymphocyte cultures, and inhibition of antibody-dependent lymphocyte-mediated cytotoxicity (10-15).

In the present report, a number of methods have been employed to study the proportion of lymphocytes having B and thymus-derived (T) cell surface markers in both peripheral blood and joint fluid. Particular attention has been directed to evaluating antilymphocyte antibodies and other factors that became apparent as sources of potential interference with the determinations of the T and B cells. Isolation procedures and analyses for surface markers have been altered to minimize these effects. In the studies on joint fluids, an unusual cell type lacking detectable surface markers was examined in further detail.

## METHODS

Patient selection and general procedures were as previously described (16). Samples of blood and joint fluid used for cell isolation were collected in preservative-free heparin; paired samples refer only to those collected at the same time. Except where noted, all joint fluids were incubated briefly with hyaluronidase (Worthington HSEP, Worthington Biochemical Corp., Freehold, N. J.) before cell isolation. Leukocyte counts were made with Türk's solution. Differential counts were made on smears stained with Wright's stain. In the case of joint fluids, differential counts were also made on similarly stained cytocentrifuged samples.

**Cell isolation.** Samples of blood or joint fluid promptly diluted with an equal volume of 4°C Hanks' balanced salt solution lacking calcium and magnesium (HBSS Ca<sup>-</sup> Mg<sup>-</sup>), and the mononuclear cell layer was obtained by room temperature centrifugation on Ficoll-Hypaque gradients (Winthrop Laboratories, Div. of Sterling Drug, Inc., New York) (17). The Ficoll-Hypaque solution was stored at 4°C until the layering. The resultant cell layer was washed three times with 4°C HBSS Ca<sup>-</sup> Mg<sup>-</sup>. Identification of phagocytic cells was routinely made by latex ingestion. Incubation was carried out for 45 min in calcium and magnesium-containing HBSS. In certain studies, to avoid warming the lymphocytes, only an aliquot was incubated with latex. In some instances whole blood or aliquots of the mononuclear cell preparation were resuspended in 50% autologous serum or fetal calf serum (FCS) and incubated within loosely packed nylon wool columns for 45 min at 37°C. Viability was assessed by trypan blue exclusion and only preparations with more than 95% dye-excluding lymphocytes were used. Samples were adjusted to 5 × 10<sup>6</sup> lymphocytes/ml in HBSS and, if necessary, stored briefly in a melting ice bath.

**Lymphocyte classification.** As a measure of the T cells, sheep erythrocyte rosettes were performed according to the method of Bentwich, Douglas, Siegal, and Kunkel (18), employing AB serum and a 5-min incubation at 37°C, but with incubation overnight at 4°C, without azide, before counting. Trypan blue was added 15 min before reading to permit the exclusion of dead lymphocytes. In certain cases before and after overnight culturing, a comparison was

made of the number of rosette-forming cells detectable after a 1-h incubation period at 4°C without the presence of AB serum. In certain experiments, an enriched population of non-T lymphocytes was obtained from the joint fluids by removing adherent cells on nylon columns and then depleting T lymphocytes after formation of sheep erythrocyte rosettes on Ficoll-Hypaque gradients. The presence of surface Ig on lymphocytes was measured by direct immunofluorescence. Rabbit antisera were raised against human IgG, IgA, and IgM monoclonal proteins and conjugated with tetramethyl rhodamine as described previously (15). They were rendered specific by Sepharose-conjugated immunoabsorbents. The specificity was verified by surface staining of monoclonal lymphoid cells and with blocking by various insolubilized monoclonal immunoglobulins. The antisera were employed as a polyvalent anti-Ig mixture. 5 × 10<sup>5</sup> mononuclear cells in 0.025 ml phosphate-buffered saline (PBS) containing 0.02% sodium azide and 2% bovine serum albumin (PBS-BSA) were reacted with an equal volume of the rhodamine-conjugated antiserum for 30 min at 4°C. The cells were washed three times with 2 ml of PBS-BSA. Fluorescein-conjugated aggregated IgG was used to identify the Fc receptors of B cells, as previously described (19).

**Cultures.** Aliquots of the latex-labeled cell isolates were pelleted and adjusted to 5 × 10<sup>6</sup> lymphocytes/ml in RPMI 1640 with 10% FCS (Associated Biomedic Systems, Inc., Buffalo, N. Y.). They were placed in T-25 flasks, gassed, and incubated at 37°C overnight in 5% CO<sub>2</sub> and 95% air. The flask was sampled at the beginning and end of the culture period and lymphocyte recovery approached 100%. Monocyte recovery was variable. Only cultures in which lymphocyte viability remained higher than 95% were used. Cells were washed three times before use.

## RESULTS

**SLE.** When lymphocytes were isolated by the procedure with cold buffers, up to 100% of the cells from certain patients had surface immunoglobulin detected by immunofluorescence. By contrast, for normal individuals studied with the same methods, only the B lymphocytes had detectable surface Ig, which was present in patterns of fine granular staining. In patients with SLE, the surface Ig often assumed different staining patterns, characteristic of the given patients on subsequent analyses. Some patients showed a very fine punctate distribution of the surface Ig present on all lymphocytes. This was best appreciated by slowly changing the focus of the microscope on different levels of the cell and was difficult to photograph. Figs. 1*a* and *b* show an intermediate pattern given by patient Es. The distribution of surface Ig is coarse and Ig was detected on all lymphocytes. Figs. 1*c* and *d* illustrate another pattern in which extremely heavy staining was found on only 10% of the lymphocytes from patient Be. This resembled the pattern given by staining with immune complexes or aggregates; Be monocytes were similarly brightly stained. 2% of lymphocytes had Ig in a capped distribution; the remainder of the lymphocytes of this patient showed very fine punctate staining. Studies on the 24 patients with SLE are

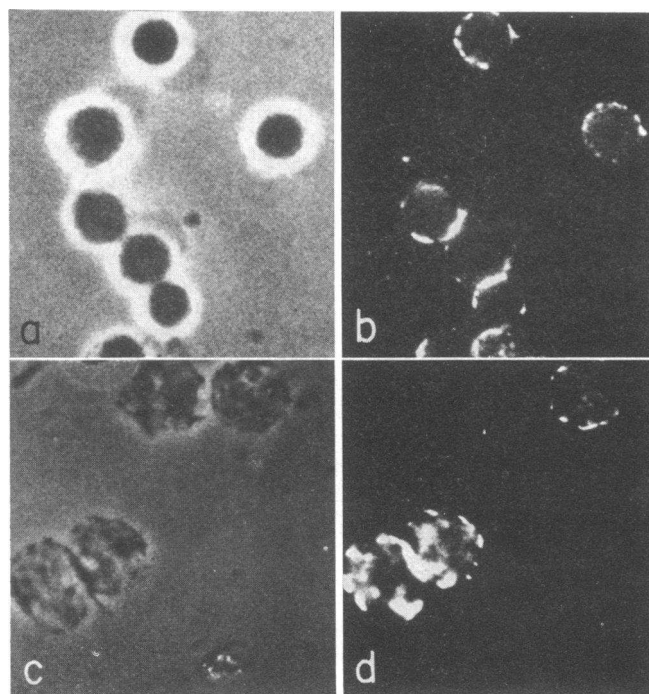


FIGURE 1 Peripheral blood lymphocytes from two patients with SLE, isolated by procedures using cold buffers, and stained for the presence of Ig on the surface membrane by rhodamine-conjugated rabbit anti-human Ig. *a*, phase contrast micrograph of lymphocytes from patient Es. *b*, fluorescence micrograph of the same field of cells showing variable staining of all cells. In some areas the surface Ig was present in sectant patches, while in others it was coarsely punctate. *c*, phase contrast micrograph of flattened lymphocytes from patient Be. *d*, fluorescence micrograph of the same field. Two cells are heavily stained with large patches similar to those given by large preformed complexes. One cell appears negative, although very fine staining was discernable on the fresh preparation.

summarized in Table I. Ig was demonstrable on more than 75% of the lymphocytes in 14 of the 24 patients. Only three patients had a value of surface immunoglobulin within the normal range and in a distribution typical of B lymphocytes.

TABLE I  
*Surface Markers on Freshly Isolated Peripheral Blood Lymphocytes*

	Surface Ig-bearing	Aggregated IgG-binding	Erythrocyte rosette- forming
	%		
Normal individuals	21.5±7.8	21.8±8.2	78.0±7.1
SLE	68.5±30.5	13.1±5.6	83.3±11.0
RA	31.4±9.0	27.2±6.7	85.1±5.1

The data in this table were obtained with the same reagents used in both normal and disease states and are meaningful for comparison. More recent values for the mean levels of surface Ig and aggregate binding in normal individuals, obtained in this laboratory with procedural modifications, are considerably lower.

Among the majority of patients who had large percentages of lymphocytes with surface Ig demonstrable after isolation in the cold, parallel isolations carried out with nylon wool or with washing steps in 20–25°C buffers resulted in a variable but considerable decrease in the number of cells with detectable surface Ig. This effect was more pronounced with 37°C buffers, as is shown in Table II. Eight patients with large percentages of lymphocytes bearing surface Ig were studied in this fashion and each showed a substantial, although variable, decrease in the percent of cells with surface Ig. To define this phenomenon more reproducibly, overnight cultures of the unstained lymphocytes were made in media containing no autologous serum. Upon retesting, the level of surface Ig was found to have substantially fallen to within or below the normal range. Representative experiments are listed in Tables III and IV. In the latter table, absolute lymphocyte counts are presented. The omission of autologous serum was important, since parallel cultures made in a medium containing autologous serum in addition to the FCS did not show a significant decrease in the percent of cells with surface Ig.

TABLE II  
Effect of Brief Incubation and Washing upon Surface Ig Detectable on SLE Patient Lymphocytes

Conditions		Ig staining		
Incubation	Washing	Di(SLE)	Ha(SLE)	Wi(normal)
°C		%		
4°C × 10 <sup>1</sup>	4°	94.2	86.4	15.1
None	37°	45.2	19.5	13.0
37°C × 10 <sup>1</sup>	37°	4.2	4.3	13.8

5 × 10<sup>5</sup> freshly isolated lymphocytes obtained with the 4°C isolation method were incubated at a concentration of 1 × 10<sup>6</sup>/ml in HBSS under the specified conditions. Washing was accomplished by adding 2 ml of PBS-BSA at the indicated temperature and promptly centrifuging at 400 g for 5 min. The cell pellets were then stained with rhodamine-conjugated anti-Ig and the Ig-bearing lymphocytes enumerated by fluorescence microscopy.

More detailed subsequent studies on these cold-reactive antibodies will be reported separately and show that the antibodies were primarily of the IgM class in most patients.<sup>2</sup> Analyses of the smaller decrease in surface Ig found in normal individuals on overnight culture show it was mainly limited to a loss of IgG-bearing lymphocytes, with the percent of cells bearing IgM or IgD remaining constant.

Several variations in the method of performing the sheep erythrocyte rosette assay were explored to obtain reproducible results. The method of using overnight incubation at 4°C with AB serum proved to be the most reliable technique. With this, the mean of the rosetting lymphocytes was 83.3 ± 11.0 (Table I). The absolute numbers of rosette-forming lymphocytes are shown for representative cases in Table IV. When rosette formation was studied under modified conditions, with a 1-h incubation at 4°C without AB serum, in those cases with high initial levels of Ig-bearing lymphocytes, values frequently as low as 20% were obtained. These rosettes were readily disrupted and difficult to enumerate. After overnight culture of the cells in medium at 37°C, the number of rosette-forming lymphocytes given by retesting with this modified method was greatly increased.

The mean percent of freshly isolated lymphocytes binding aggregated IgG was 13.1 ± 5.6 (Table I) and did not change significantly after overnight culture. This value is lower than the mean for normal individuals. Among all SLE patients, when this B cell marker is taken together with the number of rosette-forming

<sup>2</sup> J. B. Winfield, R. Winchester, P. Wernet, S. Fu, and H. G. Kunkel. 1974. Characteristics of the different types of lymphocyte antibodies found in the serum of patients with systemic lupus erythematosus. *Arthritis Rheum.* In press.

cells, a mean of 96.4% of the lymphocytes can be accounted for. 2 of the 20 patients had a marked increase in the percent of aggregate binding cells and a large number of these cells also formed sheep cell rosettes. The latter cells, giving the double reaction, were no longer present after culture.

*Patient information.* Of the 24 patients with SLE, 12 had active disease of a major organ system and were receiving more than 20 mg prednisone daily. Three were also receiving azathioprine or cyclophosphamide. Representative cases are summarized in Table IV.

The patients were arbitrarily divided into groups according to the percentage of lymphocytes bearing surface Ig. 14 patients had more than 75% of cells with surface Ig and 10 had lower percentages. 9 of the 14 patients with the higher percentage of lymphocytes bearing surface Ig and 3 of 10 patients with the lower percentages had active disease of a major organ system. The mean lymphocyte count of the 14-patient group was 1,100/mm<sup>3</sup>, while that of the 10-patient group was 1,700/mm<sup>3</sup>. The percentage of sheep erythrocyte rosette-forming lymphocytes was 83 in each group. All patients were receiving widely varying therapies. 10 of the 14 patients with elevated lymphocyte surface Ig were receiving over 20 mg prednisone daily and 2 were receiving azathioprine or cyclophosphamide. A serial study is in progress to delineate further the relationship between the indices of disease activity and the present findings.

TABLE III  
Diminution in Surface Ig Detectable in RA and SLE Lymphocytes upon Overnight Culture

Patient	Ig-bearing lymphocytes	
	Freshly isolated	After culture
	%	
ES (SLE)	98	8
OD (SLE)	62	3
Wo (SLE)	52	2
Mc (SLE)	27	3
Ro (RA)	88	6
Po (RA)	43	4
Lo (RA)	32	3
Sz (RA)	14	1
Wi (normal)	15	11
De (normal)	21	17
St (normal)	9	8

Freshly isolated lymphocytes were placed in RPMI 1640 with 10% FCS at a concentration of 5 × 10<sup>6</sup>/ml. They were incubated overnight in 5% CO<sub>2</sub> at 37°C. All recovery and viability exceeded 95%. Aliquots were then stained with rhodamine-conjugated anti-Ig and the Ig-bearing lymphocytes enumerated.

TABLE IV

Patient information		Laboratory*			Precipitins		Number of lymphocytes per mm <sup>2</sup> †			
							Total	Rosette-forming	Surface Ig before culture	Surface Ig after culture
Age, sex, duration of illness, and clinical salients	Therapy	ANA	RF	CH <sub>50</sub>	Clq	Ha				
<b>SLE</b>										
Be. 30 yr, female, 6 yr active cranial and peripheral neuritis, arthralgias	Prednisone, 20 mg	3	0	80	0	0	1,180	1,000	1,180	94
Di. 50 yr, female, 27 yr Active discoid skin lesions and mild nephritis	Plaquenil, Prednisone, 20 mg	3	0	131	1	0	1,800	1,580	1,710	54
Es. 19 yr, female, 2 yr malar rash and active nephritis	Prednisone, 50 mg, alternate days	1	0	74	1	0	700	405	690	56
Ham. 58 yr, female, 15 yr severe thrombocytopenia (active)	Prednisone, 25 mg	3	0	115	0	0	3,800	3,300	2,200	150
Har. 56 yr, female, active myositis and peripheral neuritis	Prednisone, 30 mg	3	0	91	0	0	1,000	870	880	100
It. 50 yr, female, 18 yr arthralgias fever and alopecia (active)	Prednisone, 20 mg alternating with 5 mg	2	0	180	0	0	1,400	1,260	1,400	56
Od. 40 yr, female, 15 yr active nephritis	Cyclophosphamide, 150 mg	4	0	105	1	0	780	624	480	23
Sa. 50 yr, female, 13 yr malar rash fatigue and arthralgias (inactive)	Prednisone, 15 mg	2	0	99	0	0	3,450	3,240	620	140
Wo. 12 yr, female, 4 yr active nephritis and thrombocytopenia	Azathioprene, 75 mg Prednisone, 25 mg, alternate days	3	1	220	0	0	1,700	1,610	890	34
<b>RA</b>										
Co. 63 yr, male, 15 yr active effusive synovitis with destruction of joints. No nodules	Gold salt PB JF	tr 3	2 2	175 <6	0 4	3 4	1,000 100	800 4,236	480 565	30
Fa. 64 yr, female, 27 yr moderate synovitis with marked destruction. No nodules	Prednisone, 5 mg PB JF	0 0	2 1	250 <6	0 2	0 2	920 5,520	710 3,920	220 330	9
Go. 56 yr, female 21 yr active synovitis with marked destruction. Occasional nodules	Penicillamine, 1.5 g PB JF	0 0	4 4	250 16	0 1	0 2	2,900 200	92 142	226 12	145
Lo. 62 yr, female, 26 yr active synovitis with destruction numerous nodules vasculitis, pericarditis, pulmonary fibrosis	Prednisone, 2.5 mg PB JF	1 3	3 4	145 <6	0 4	3 4	700 2,900	640 2,440	234 201	21
Ro. 77 yr, female, 7 yr, active synovitis with destruction, splenomegaly and nodules	Prednisone, 5 mg PB JF	1 3	4 4	136 17	0 3	2 4	1,100 8,800	91 5,900	88 328	6
Sa. 70 yr, male, 5 yr mild synovitis, Felty's syndrome, with pancytopenia	Prednisone, 10 mg PB	3	4	184	0	2	200	176	100	20

\* Antinuclear antibodies (ANA) were measured on human liver sections. Rheumatoid factor (RF) was measured in a microprecipitation assay with aggregated IgG. CH<sub>50</sub> was measured by the method of Kent and Fife. Range of normals 150–250 U/ml. Precipitin tests were performed according to (16) using purified Clq or a RF reagent, Ha. Results were scored semiquantitatively. Peripheral blood (PB) or joint fluid (JF) studies were done on paired samples obtained at the same time.

† Total lymphocyte counts were computed from differential count and leukocyte count. The number of rosette-forming lymphocytes were measured with AB serum and overnight incubation at 4°C. Surface Ig before culture refers to freshly isolated cells obtained by using 4°C buffers. Cells were placed in overnight culture at 37° in RPMI 1640 — 10% FCS washed and assayed for surface Ig by immunofluorescence. The joint fluid lymphocytes were not cultured overnight.

*RA.* Lymphocytes from some patients with RA had extreme amounts of Ig detectable on the cell surface, as in the findings in patients with SLE when the same method of cold isolation was used. This Ig also often differed considerably from the surface staining pattern characteristic of normal B lymphocytes. The patterns

of surface Ig staining varied from fine through coarse asymmetrical patching to definite dense cap formation. The latter is illustrated in Fig. 2 *a* and *b*. Of 44 patients studied, 3 had Ig detectable on over 90% of lymphocytes, while 18 others had levels of surface Ig-bearing lymphocytes above the upper limit of normal. The

mean value of surface Ig for all patients was 31% (Table I), while the lowest level encountered was 12%. Many patients had very bright staining of from 15 to 25% of the lymphocytes; Fig. 2c and d is an example of this pattern. Lymphocytes from 11 patients were cultured overnight. A marked decrease in the percent of Ig-bearing cells was found in each case, as shown by the examples in Table III. In three instances, as shown by patient Sz, the initial Ig was not elevated, yet after culture the decrease in Ig exceeded that seen for normal subjects.

The number of rosette-forming lymphocytes determined by overnight 4°C incubation and AB serum was  $85.1 \pm 5.1$  (Table I). The modified rosette system with 1-h incubation at 4°C and without AB serum gave markedly lower numbers of rosette-forming lymphocytes in some freshly isolated samples. When retested by the same method after short-term culture, the number of rosette-forming lymphocytes appreciably increased and often became equal to the numbers obtained by the method with 4°C overnight incubation.

Aggregated IgG binding was found on a mean of 27.2% of lymphocytes (Table I). After overnight culture, this fell to a mean of 16.3%; and it was clear that in some of the cases, the culture removed material, prob-

ably rheumatoid factors, which caused this binding of aggregated IgG. The high levels of aggregated IgG binding were not found in normal individuals and were uncommon in SLE patients. After overnight culture, cells of four patients had proportionally smaller declines in aggregate binding than the more marked decrease in surface Ig. For example, Po in Table III gave 32% aggregate binding before culture, and 23% after culture, even though the Ig-bearing cells fell from 43% to 4% after culture; 80% of the cells formed rosettes. Thus in this case, the major proportion of the non-rosette-forming cells did not have detectable surface Ig but bound aggregated IgG.

#### Joint fluid lymphocyte populations

*RA.* The synovial fluids of 23 patients were studied to enumerate lymphocytes bearing T or B cell markers. Table V shows the mean values obtained and includes several representative individual patients. The mean percentage of lymphocytes with detectable surface Ig was 5.8 and the range 1–15. 6.6% of lymphocytes bound aggregated IgG (range 1–23). In three patients, the number of lymphocytes binding aggregated IgG was more than two times those bearing surface Ig, and this did not diminish significantly after culture. Patient Po

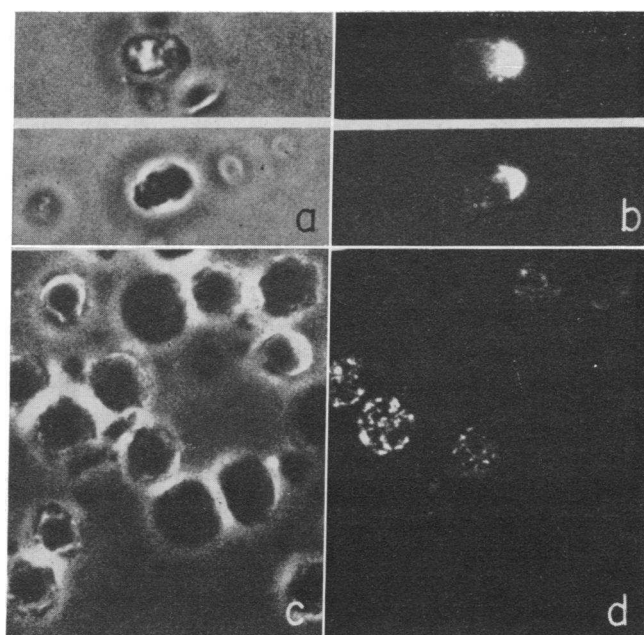


FIGURE 2 Peripheral blood mononuclear cells from two RA patients stained for the presence of Ig on the surface membrane by rhodamine-conjugated rabbit anti-human Ig. *a*, phase contrast micrograph of selected cells from patient Ro. *b*, fluorescence micrograph of the same fields showing cap formation. Small amounts of surface Ig staining persist around the periphery of the cell. *c*, phase contrast micrograph of cells from RA patient Mo. *d*, fluorescence micrograph of the same field showing unusually bright staining of a minor proportion of the cells.

TABLE V  
Joint Fluid Lymphocyte Populations

Patient	Bearing surface Ig	Binding aggregated IgG	Erythrocyte rosette forming
		%	
Co (RA)	4	3	72
Go (RA)	6	7	71
Po (RA)	3	10	87
Tu (RA)	3	1	80
Ga (SLE)	10	12	92
Rheumatoid arthritis patients ( $n = 23$ ), mean $\pm$ SD	5.8 $\pm$ 3.1	6.6 $\pm$ 4.7	75 $\pm$ 8.4

(Table V) is representative of these. The mean percentage of lymphocytes forming rosettes with sheep erythrocytes was 75 (range 55–87). The rosettes, after 1 h of incubation at 4°C, were distinctive, in that the erythrocyte often clustered densely around the central lymphocyte, forming a “morula” pattern. Their percentage was not further increased by addition of AB serum or overnight incubation. Latex-ingesting cells were routinely excluded from analysis.

In contrast to peripheral blood (Table I), it was not possible to account for all lymphocytes in most RA joint fluids by calculating the sum of those lymphocytes forming rosettes (T cells) with either those bearing surface Ig after culture or those binding aggregated IgG (B cells). Thus up to 32% of joint fluid lymphocytes belong to a population that failed to manifest any of the assayed markers. In the case of 3 of the 23 fluids, results similar to those from Po in Table IV were obtained; the sum of rosette-forming cells plus those binding aggregated IgG accounted for most of the lymphocytes, despite the lowered percentage of lymphocytes with surface Ig. Thus in these fluids the second major type of lymphocyte contained a receptor for aggregated IgG but lacked surface Ig.

To delineate further the population of RA joint fluid lymphocytes lacking recognizable B or T cell markers, additional analyses were made on joint fluids from Co and Go. Monocytes were eliminated by nylon wool purification or marked by latex ingestion. Rosetting lymphocytes were selectively depleted through preparative rosette formation. The resulting interface population of cells in patient Co contained 87% nonrosetting, non-Ig-bearing cells, with 5% forming rosettes and 8% having surface Ig. This T cell-depleted lymphocyte population was examined for cell morphology with Wright's stain after cytocentrifugation. The predominant cell was a small lymphocyte, with 18% large lymphocytes. One apparent blast was seen among 480 cells. An aliquot of

this preparation was prepared for transmission electron microscopy and examined by Dr. Steven Douglas. All cells resembled typical lymphocytes; no synovial lining cells or phagocytic cells were encountered. The cells remained viable in cultures containing FCS but did not resynthesize surface Ig.

**Other joint diseases.** Synovial fluids from three patients with SLE were studied. Surface Ig found was on 10, 18, and 28% of the lymphocytes. A representative case is included in Table IV. This patient had 18,000 lymphocytes/mm<sup>3</sup> of joint fluid. The other patients had less marked lymphocyte elevations. Nine additional fluids were obtained from patients with various diseases, including septic arthritides (staphylococcal and gonococcal), acute gout, and ankylosing spondylitis. The percentage of lymphocytes having surface Ig ranged from 12 to 32.

**Patient information.** The patient population with RA represented a group selected with considerable bias towards inclusion of patients with severe joint or extra-articular manifestations. Most of the patients had long-standing disease and had previous courses of gold salt therapy. All were receiving drugs of the analgesic-anti-inflammatory class, six were receiving gold salt therapy, and eight were receiving 5 mg prednisone daily, and one 10 mg daily. Representative cases are summarized in Table IV. Grouping the patients according to high or low levels of peripheral blood lymphocyte surface Ig did not distinguish a particular group of clinical or laboratory findings.

8 of 11 patients studied showed an increased concentration of lymphocytes in the joint fluid relative to that found in the peripheral blood. The mean ratio of these concentrations was 4:1. Examples are shown in Table IV. In 11 of 13 joint fluids, the polymorphonuclear leukocyte was the preponderant cell. Fluid Co with 66% lymphocytes and a leukocyte count of 10,700/mm<sup>3</sup>, and fluid Tr with 92% polymorphonuclear leukocytes and a leukocyte count of 35,000/mm<sup>3</sup> represented the extreme situations found. Yet in each there was enrichment of lymphocytes in the joint fluid, of which approximately one-fourth lacked detectable surface markers. The patients receiving low-dose prednisone therapy or gold salt therapy were not distinguishable from other patients on the basis of number of lymphocytes or the proportion of cells with T or B markers. Patient Go, described in Table IV, was receiving 1.5 g penicillamine, daily for 4 mo and exhibited the lowest joint fluid lymphocyte count among all the patients. This patient did not have an increase in surface Ig on the peripheral blood lymphocytes.

## DISCUSSION

Through the use of immunofluorescence techniques, abundant Ig was demonstrated on a large proportion

of peripheral blood lymphocytes from certain patients with SLE and RA. In the normal individual, similar methods demonstrate surface Ig that is an intrinsic component of the B lymphocyte plasma membrane. This reaction is routinely used as a marker of the B lymphocyte population (1, 2). Two lines of evidence support the interpretation that the markedly increased surface Ig found in this study had a different origin from the Ig intrinsic to the B lymphocyte surface. First, the sum of lymphocytes that have detectable surface Ig plus the lymphocytes possessing the T cell attribute of forming rosettes with sheep erythrocytes greatly exceeded 100%. Thus a significant proportion of the T lymphocytes have readily detected surface Ig, a finding contrary to the situation in normal individuals (1, 2). Secondly, the excess surface Ig was labile and rapidly lost if the cells were cultured at 37°C in the absence of autologous serum. Evidence was obtained that this increased surface Ig arose from several sources.

Most of the additional surface Ig was antibody directed to autologous plasma membrane components. Support for the antibody nature of the surface Ig was provided by experiments in which lymphocytes that had previously lost the excess surface Ig regained it after reincubation in autologous serum. In addition, a parallel study has shown that these sera contained antibodies that were similarly reactive with both normal lymphocytes and the autologous lymphocytes. This reactivity was demonstrated both by fluorescence microscopy and by cytotoxicity. Fluorescence microscopy proved to be considerably more sensitive than cytotoxicity as a means of recognizing these antibodies. These results and the further characterization of the antibodies as to class, specificity, and physical properties will be the subject of a separate report.<sup>3</sup> Thus these antibodies are presumably related to those detected previously by lymphocytotoxicity (10-12) and mixed leukocyte culture inhibition (13, 14, 20).

An interesting property of a large proportion of the antibodies encountered in the present study is that, while firmly adherent at 4°C, they are readily lost on incubation at higher temperatures, like cold agglutinins or cold-reactive rheumatoid factors (21). Thus they may be considered cold-reactive antilymphocyte antibodies. A lower temperature optimum of 15°C has previously been described for the lymphocytotoxins of SLE (10); however, their activity is still detectable at 37°C (12).

The use of isolation steps carried out in the cold favors the uptake and avid adherence of available cold-reactive antilymphocyte antibodies. The weaker interactions of these antibodies at intermediate or at body temperatures would result in their variable adsorption and elution during the preparative procedures. The cold isolation procedure thus may be used to give an

estimate of the interfering substances present in the serum but an equivalent estimate may be made by reincubating the cells isolated under warm conditions in autologous serum at 4°C and carrying out subsequent steps in the cold. This variation also permits prior labeling of monocytes with latex and simplifies subsequent analyses. It was necessary to allow the cold-reactive antibodies and other interfering substances to leave the lymphocyte surface before a true picture could be obtained of the surface markers characterizing the different lymphocyte populations. Isolation and washing steps carried out at 37°C avoided much of the adsorbed surface Ig; however, there were some antibodies still detectable in certain cases. Overnight culture at 37°C in the absence of autologous serum permits shedding of surface antigens along with the accompanying antibody (13) and this procedure, which combines elution at 37°C and shedding, proved to be the best in the present study. Other mechanisms may also contribute to ridding the lymphocyte surface of foreign substances, as summarized by Unanue and Karnovsky (22).

Evidence was obtained indicating that rheumatoid factors also may be involved in the lymphocyte surface reactions of certain patients with RA. Since some of the antilymphocyte antibodies are of the IgG class (14),<sup>3</sup> cells coated with these antibodies could be targets for reactions with rheumatoid factors. The presence of rheumatoid factor-antilymphocyte antibody complexes would be an explanation for the high binding of aggregated IgG by isolated lymphocytes from certain patients. This involved T cells, at least in part, and diminished after culture. It is likely that the lymphocyte capping is induced by a similar interaction of rheumatoid factors with IgG antilymphocyte antibody. Patching and cap formation was induced by adding isolated rheumatoid factors to a serum containing IgG antilymphocyte antibodies, which by itself gave a fine punctate pattern of staining on the lymphocyte surface.

The number of T lymphocytes was slightly increased in both diseases, as determined by erythrocyte rosette formation carried out with overnight incubation at 4°C. Performed in this manner, inhibition, presumably due to the antilymphocyte antibodies, is minimized. However, when rosette formation was tested with short periods of incubation and without the addition of AB serum, as in some laboratories, much lower levels of rosette-forming lymphocytes were found in many freshly isolated preparations. It was apparent that interfering antibodies were involved in this system as well. These effects were not apparent with lymphocytes that had been put into overnight cultures.

In a few joint fluid and peripheral blood samples from RA patients, cells bearing the aggregate receptor were found at more than twice the level of Ig-bearing B cells.



These did not diminish significantly after overnight culture and they accounted for the major portion of all nonrosetting lymphocytes in these cases. This population of lymphocytes could represent an unusual type of B cell. Another possibility is that this cell may be related to the K cell responsible for antibody-mediated cytotoxicity (7, 23, 24). This cell may simulate an Ig-bearing B lymphocyte by the uptake of circulating immune complexes on its surface and represent the bright-staining subpopulation of lymphocytes that was sometimes observed.

The antilymphocyte antibodies, along with rheumatoid factors and IgG complexes, may thus cause considerable interference with the assessment of T and B cells in freshly isolated lymphocytes. It is likely that high percentages of B cells reported in some previous studies were due to minor variations in technique that favored persistence of these interfering substances, while other studies showing more normal levels were carried out under conditions favoring removal of the antibodies.

The initial report by Papamichail et al. on surface Ig in patients with RA describes high levels of Ig staining and includes an illustration of patched and capped lymphocytes (3). This presumably represented some coating of the lymphocytes by antilymphocyte antibodies. Similarly, Messner, Lindstrom, and Williams (6) have commented on the significant disproportion between the relatively elevated lymphocyte surface Ig in patients with SLE and the disproportionally lowered number of cells bearing receptors for C3. The low level of cells bearing the receptor for C3 is in agreement with the low numbers of B cells found in the present study, as determined both by the number of cells with persistent surface Ig after overnight culture as well as by the initially low percentage of aggregate binding cells. Johnson, Zeider, and Jacobs have recently reported finding in a considerable number of patients with SLE or RA that, in agreement with this study, the sum of rosette-forming lymphocytes and those having surface Ig greatly exceeded 100%, indicating the presence of antilymphocyte antibodies, and that true B lymphocytes occurred at lower levels (25). The interesting observation by Yu et al. (26) of changes in the composition of B and T cell population with prednisone therapy is not an explanation for the findings in the present study, since by several criteria, including aggregate binding, those authors show that they were dealing with a true B cell population and not with the simulation of B cells by antibody coating.

The question of whether any of the cold-reactive antibodies are attached to the lymphocytes *in vivo* remains to be determined. The situation in the body is complicated by two opposing factors: greatly lowered antibody

avidity at 37°C and the presence of significant excess concentrations of antibody in the plasma. The extent to which the elevated antibody concentration compensates for the loss of avidity is unknown. Certainly, because of the dissociating conditions of lymphocyte isolation, washing, and staining, most of the antibody found on the cells by immunofluorescence was acquired secondarily through the manipulations at lower temperatures and reflects antibodies with sufficient cold-enhanced avidity to withstand the dissociating preparative procedures. The situation might be analogous to that occurring in cold agglutinin disease, where the vast bulk of the cold agglutinins do not react with the patients' red cells *in vivo*, but enough of them do to cause hemolytic anemia. Some evidence was obtained in the present studies that low concentrations of the antibodies, particularly of the IgG type, might be adsorbed even at body temperatures. Further studies are required on these points, particularly in distinguishing the antibodies with cold-enhanced reactivity from IgG antibodies, which also are known to be present in some SLE patients through their activities at 37°C (10, 19). It is possible that the antilymphocyte antibodies contribute to the lymphopenia by shortening the life-span or altering the circulatory pattern of the cells. But a potentially more significant effect of the antibodies is that they might interfere with the interplay of lymphocyte populations that occur in the normal immune response. By this means they may further the abnormal immune state that led to the production of the antilymphocyte antibodies. Another potential effect of this variety of autoantibody is that it could contribute to immune complex disease by combining with shed lymphocyte surface components that may be present in the circulation.

In the joint fluids of RA patients, an unusual cell population was evident that lacked all assayed markers. These cells formed the second major lymphocyte population, greatly exceeding the number of typical B cells in the synovial fluid. By morphologic and ultrastructural criteria, the cells appeared to be lymphocytes and their unusual properties remained stable in culture for 24 h. Cells such as these have been termed "null cells" and represent a significant population in the mouse spleen, particularly in the NZB/W hybrid animals (27, 28). In joint fluids, this may be a precursor cell that reflects the characteristic lymphoproliferation in the synovium or it could be a mature lymphocyte modulated by the intense immunologic reactivity of the synovial fluid. Joint fluid lymphocytes are known to respond poorly to phytohemagglutinin (29) and function poorly as both stimulator and responder in the mixed lymphocyte culture (5). The rosette-forming lymphocytes in the joint fluid were distinctive in that they were surrounded with

an exceptionally dense cluster of erythrocytes. Even after overnight culture, the peripheral blood lymphocytes failed to show this morula-like pattern. B lymphocytes occurred at very low percentages, though in some patients the absolute number of B lymphocytes was equivalent to the number in peripheral blood. The occurrence of low percentages of B lymphocytes in joint fluids has also been recently reported by Frøland (30). In contrast to the findings in peripheral blood, antilymphocyte antibodies were not detected on the joint fluid lymphocytes. One explanation for this could be that the antilymphocyte antibodies are present in the synovial fluid in the form of immune complexes coupled to antigenic fragments shed from the lymphocyte surface. Efforts are currently underway to determine if such postulated complexes account for a portion of the complexes previously observed in these joint fluids.

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