Studies of T- and B-Lymphocytes in Patients with Connective Tissue Diseases

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ABSTRACT Peripheral blood lymphocytes from normal subjects as well as patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and active tuberculosis were studied for the relative distribution of bone marrow-derived lymphocytes (B-cells) and thymic-derived T-cells. B-cells were identified by direct immunofluorescence of surface Ig markers; T-cells were studied using rabbit antisera to pooled human fetal thymocytes absorbed with chronic lymphatic leukemia lymphocytes as a source of B-cells. In normal subjects, the sum of percentages of peripheral blood lymphocytes staining for surface Ig (B-cells) plus the percentage of cells staining with the absorbed antithymocyte antiserum closely approximated 100%. The mean value for percent B-cells among 51 normals tested was 22.9%±7.1; mean T-cells value was 75.3±13.95%. T-cell-specific antiserum stained 18% of normal human bone marrow lymphocytes, 42.5% of lymphocytes from normal spleens, and 98% of cells obtained from thoracic duct drainage of patients with RA. Specificity of antihuman thymocyte antiserum appeared to depend on the use of living cells.

When patients with RA were examined, a wide range (14–98%) of peripheral blood T-cell values was found. Values for low percentages of peripheral blood T-cells appeared to correlate to some extent with severe clinical disease. In 11 of 36 RA patients, the sum of identifiable B- plus T-cells accounted for only 34–55% of peripheral blood lymphocytes. The identity of the remaining "null" cells could not be identified.

3 of 24 SLE patients studied showed low percentages of peripheral blood T-cells, but no correlation could be drawn between T- to B-cell ratios and clinical disease activity. Among 21 patients with active tuberculosis, one had a low value for identifiable T-cells. No significant differences from normals in range or proportion of B-cells was identified in patients with active tuberculous infection.

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INTRODUCTION

Connective tissue disorders such as rheumatoid arthritis (RA)[1] or systemic lupus erythematosus (SLE) are characterized by histopathologic evidence of lymphocytic infiltrations within many of the basic tissue lesions. For well over a decade investigative focus in these disorders has revolved around description and characterization of tissue-reacting humoral antibodies and so-called autoantibodies as being important to the disease processes (1–10). Recently more attention (11–13) has been directed at cells prominently involved in the tissue lesions of RA and SLE—the lymphocytes—and their possible contribution to the pathogenesis of such connective tissue disorders. Immune lymphocytes have been characterized as belonging to two fundamental classes: (*a*) B-cells or lymphocytes bearing readily detectable surface immunoglobulin determinants and involved in the production of antibody and (*b*) T-cells derived from the thymus, bearing no readily detectable surface immunoglobulins and committed to cell-mediated immune reactions. Since little is understood of the precise effector systems involved in actual tissue destruction in patients with connective tissue disease, it seemed possible that a careful study of the peripheral blood and tissue distribution of T-cells and B-cells in these disorders might be illuminating. Clear-cut identification of peripheral blood B-cells is now possible through the use of specific immunofluorescent antisera to Ig and enu-

[1] *Abbreviations used in this paper:* ARAHT, absorbed antihuman thymocyte antisera; B-cells, bone marrow-derived lymphocytes; CLL, chronic lymphatic leukemia; HBSS, Hank's-balanced salt solution; RA, rheumatoid arthritis; SLE; systemic lupus erythematosus; T-cells, thymic-derived lymphocytes.

meration of cells containing surface Ig markers (14–23). In mice T-cells have been identified using T-cell or θ-antigen-specific alloantisera produced by immunizing one strain of mice with lymphoid tissues or thymus of another strain (24–27). Anti-θ-specific antisera have also been recently produced by heteroimmunization with mouse brain (28, 29). Preliminary reports (30) indicate that manufacture of T-cell-specific antisera to human thymus-derived cells bearing what may be roughly equivalent to the mouse θ-marker may be feasible. In this report we describe production of antisera appearing to show some degree of human T-cell specificity. Using these immunologic techniques, studies of peripheral blood distribution of T-cells are described with particular reference to SLE and RA. Our studies appear to indicate some degree of subclass (T-cell or B-cell) heterogeneity which will certainly eventually be important for a clear understanding of these diseases.

METHODS

Clinical material. 57 normal subjects ranging in age from 21 to 50 yr provided the peripheral blood lymphocytes used in our studies. 70 patients with active RA and 24 with SLE were derived from the out-patient and in-patient populations of the Bernalillo County Hospital or Veterans Administration Hospital, Albuquerque, N. M. Clinical activity of disease in individual patients was graded from 1+ to 4+ on the basis of independent review of clinical records without concurrent knowledge of the results of the special studies done. American Rheumatism Criteria for clinical staging of RA were used; in addition the clinical activity of patients with SLE was recorded on the basis of degree and severity of system involvement along with results of laboratory indicators of disease activity such as serum complement activity, leukopenia, or microscopic examination of urine sediment.

Besides the normal subjects studied, a second control group of 21 patients under treatment for active pulmonary or disseminated tuberculosis was also included. The majority of these patients had active moderate or far-advanced tuberculous infection.

Finally, because the focus of the study involved attempts to ascertain possible subtle abnormalities in peripheral blood lymphocyte populations of patients with connective tissue diseases, a small group of five patients with Felty's syndrome associated with RA were also included.

Identification of B-cells in peripheral blood. Peripheral blood lymphocytes were collected from blood drawn in heparinized syringes and cells separated using a Ficoll-Hypaque gradient as previously described (31, 32). Such cells consisted of 90–95% lymphocytes and 5–10% monocytes, as assayed by differential counting of Wright's stained smears. In some instances when the percentage of monocytes was large, e.g. $>3\%$, polystyrene beads were added to lymphocyte populations and monocytes thereafter readily identified during immunofluorescent procedures after phagocytosis of these particles. The direct immunofluorescent technique originally described by Pernis, Forni, and Amante (15) and adapted by Rabellino and co-workers (18–20) was used to enumerate relative percentages of lymphocytes bearing γG, γA, γM, or κ- and λ-light chain markers. The total number (γG + γA + γM) of cells per 100 was assigned as the percent of B-lymphocytes bearing detectable Ig. As a check on the accuracy of this system, lymphocytes were also studied using conjugated, purified specific rabbit antisera to κ- or λ-light chains, and the total percentage of cells staining for γG + γA + γM compared with the total staining for κ and λ. In general reasonably good agreement was noted between total numbers of cells obtained by sums of γG + γA + γM and that seen by adding total percentages of cells stained for κ- and λ-determinants. It was recognized that some B-cells might contain surface Ig determinants of γD or γE type. Accordingly 20 individual normal peripheral blood-lymphocyte samples were examined for total percentages of cells staining for γD and γE using immunoabsorbent column-purified-specific antibodies and the same procedure as had been used for γG, γA, and γM. The average percentage of total peripheral lymphocytes staining for γE plus γD in the sample of normal subjects studied was 0.75%. For the sake of simplicity and because of limitations in supply of antiserum, B-cell staining for γD or γE was omitted from subsequent calculations.

In previous studies by others it had been noted that percentages of B-cells identified as sums of γG + γA + γM surface-staining lymphocytes correlated well with the proportion of cells staining with anti-$F(ab)_2'$ antiserum (33). These findings along with our own previous observations (34) appeared to indicate that individual normal human peripheral blood B-lymphocytes usually contained but one predominant class of surface Ig. In general, the observations recorded here appeared to be compatible with this interpretation; however, because several striking exceptions presented themselves during the course of our studies, particularly in patients with SLE, an attempt was made to examine the question of multiplicity of surface Ig classes on single individual lymphocytes using double staining with fluorescein-conjugated anti-IgM or anti-IgA and rhodamine-conjugated anti-IgG.

Another method for enumeration of B-cells was attempted using an entirely different approach. Antisera were raised in rabbits against lymphocytes from eight individual patients with chronic lymphatic leukemia CLL). These cells were chosen since work by several groups of investigators (23, 35) has shown the vast majority of leukemic cells from such patients are probably B-cells, with often more than 90% of such leukemic cells containing surface Ig markers. Anti-B-cell or CLL antisera absorbed with fresh human thymocytes showed no apparent specificity for B-cells by cytotoxicity or immunofluorescence with several different fresh individual human peripheral lymphocyte suspensions, and therefore could not be used as an additional tool to enumerate or identify B-cells.

Identification of T-cells. Because the original mouse anti-θ-antisera had been produced in different strains of mice (25–27), attempts were made to simulate this situation by an 8 wk course of immunization of chimpanzees with a pool of fresh thymocytes from four 20- to 30-wk human fetuses obtained after therapeutic abortion or hysterotomy. Such chimpanzee antisera showed striking indirect immunofluorescence of 95–100% of peripheral blood lymphocytes from six normal human donors of varying HL-A type. However, attempts to absorb the chimpanzee antithymocyte antisera with relatively pure populations of B-cells (CLL cells containing 70–95% lymphocytes with surface immunoglobulin determinants) produced serial complete loss of indirect immunofluorescence when tested against normal peripheral blood lymphocytes. The absorptions of chimpan-

zee antihuman thymocyte antiserum were performed serially five times using 1 ml of antiserum and 60×10^6 CLL cells. Control serum was diluted to match the final total volume of the CLL-absorbed antithymocyte antiserum. Progressive loss of percentage of indirect immunofluorescent staining of normal peripheral blood lymphocytes is shown in Fig. 1. A similar diminution in lymphocytotoxic reactivity was noted in chimpanzee antihumanthymocyte antisera after serial absorption with several different individual CLL cell samples.

By contrast, rabbit antisera prepared by injection of 60×10^6 human thymocytes mixed with complete Freund's adjuvant given subcutaneously once every 2 wk for 6 wk provided a workable reagent. Five serial absorptions of rabbit antiserum using CLL cells for each absorption showed no further loss of percentage of normal peripheral blood lymphocytes staining after three absorptions. Repeated experiments indicated that after the third absorption with CLL cells, 60–85% of the test normal lymphocytes continued to show indirect immunofluorescence. Representative results are shown in Fig. 1.

RAHT sera were absorbed as noted above using five serial absorptions of 60×10^6 CLL cells/ml of undiluted rabbit antiserum. Each absorption was performed by incubating CLL cells with antiserum for 1 h at 37°C followed by centrifugation and subsequent absorption and centrifugation off of each fresh portion of CLL cells used for serial absorptions. Since each absorption of antiserum was performed using the 60×10^6 CLL cells in 0.1–0.2 ml of suspending buffer, it was estimated that five serial absorptions diluted the original RAHT antiserum between 1:1.5 and 1:2. CLL lymphocytes were used from four different patients with high percentages of cells showing surface Ig (70–95%) and results using such absorbed antisera were equivalent and comparable. After five absorptions absorbed antihuman thymocyte antisera (ARAHT) did not show indirect staining of test CLL cells showing surface Ig although in some instances 10–20% of CLL cells without detectable surface Ig showed faint staining. In the studies reported here antisera from two individual rabbits were used which had been obtained at two separate bleedings 6 wk and later 2 mo after initiation of immunization. There was no detectable difference or variation in the specificity of these two rabbit antisera, both of which showed indirect immunofluorescent staining of lymphocytes detectable to titers of 1:15–1:20; in all studies reported here, absorbed antisera (ARAHT) were used in a dilution of 1:2 to insure clear identification of staining cells during counting.

The actual indirect staining procedure employed lymphocytes isolated by the Ficoll technique and washed twice with Hank's balanced salt solution (HBSS). One drop of ARAHT diluted 1:2 was added to 1×10^6 lymphocytes in 0.1 ml HBSS and the mixture allowed to stand at room temperature (22°C) for 30 min with gentle shaking every 10 min. The cell-antiserum mixture was then washed twice with HBSS and resuspended in 0.1 ml of HBSS. A drop of fluorescein-conjugated goat antirabbit IgG diluted 1:2 (Shwarz Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.) was then added and the mixture allowed to reincubate at room temperature for 30 min again with gentle shaking every 10 min. After rewashing twice with HBSS, the cells were resuspended in 0.1 ml of HBSS and examined under fluorescent microscopy. Before use goat antirabbit IgG was absorbed with lyophilized mouse liver powder. Fluorescent microscopic examinations employed a Zeiss ultraviolet microscope (Carl Zeiss, Inc., New York) equipped with an HBO 200 mercury lamp and using a BG 12 filter.

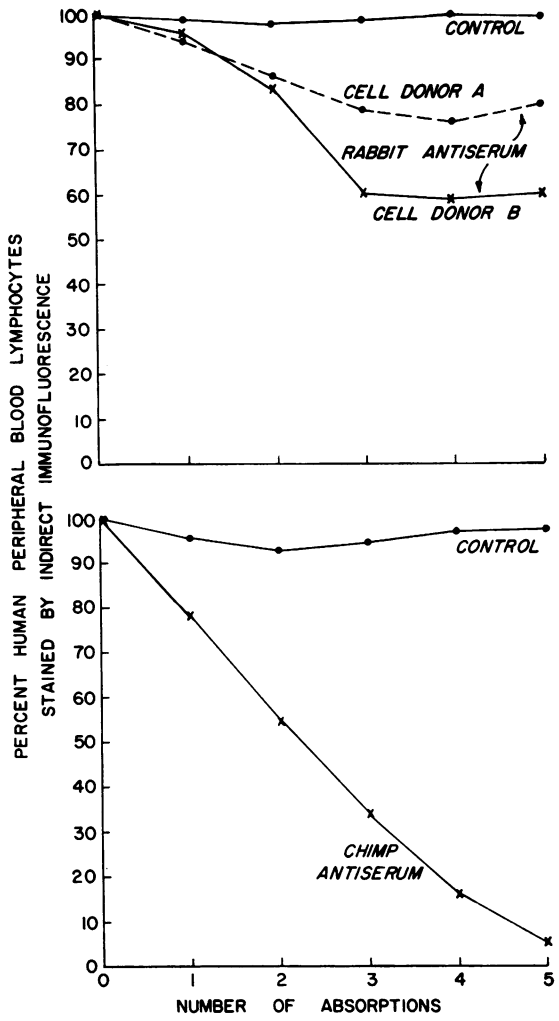

FIGURE 1 Results of five serial absorptions of chimpanzee antihuman thymocyte antiserum (ACAHT), below, and rabbit ARAHT, above, when 1 ml of antiserum was absorbed with 60×10^6 CLL cells serving as a source of B-cells. Each absorption employed 60×10^6 CLL cells, and absorbed supernate was tested with living normal human peripheral blood lymphocytes in an indirect immunofluorescence test system using fluoresceinated goat antirabbit or rabbit antichimpanzee antiserum. It can be seen that with each absorption activity against normal human lymphocytes progressively declined in the chimpanzee antiserum, whereas after the third absorption of the rabbit antihuman fetal thymocyte antiserum, the antiserum continued to react with from 58 to 80% of normal peripheral blood lymphocytes. Results with two normal human cell donors (A and B) are shown. Average or mean percentage of peripheral blood lymphocytes stained by absorbed rabbit ARAHT in 51 normal cell donors was 75.3%±13.95.

When double-staining procedures were attempted, differentiation of green, red, and mixed staining was made using sequential observation with Kodak Wratten filter 58 (Eastman Kodak Co., Rochester, N. Y.) (for green com-

TABLE I

Immunofluorescent Data on Percent of Lymphocytes Staining with Rabbit Antihuman Fetal Thymocyte Antiserum Absorbed with CLL Cells

Test lymphocytes derived from:	No. Individual samples tested	Percent positive by immunofluorescence
		%
Normal human peripheral blood	51	range 43–95 mean 75.3
Normal human bone marrow	4	range 8–26* mean 18
Normal human spleen	2	range 35–50 mean 42.5
Human thoracic duct drainage (Rheumatoid arthritis)	3	range 95–99 mean 98

* Four normal subjects had bone marrow aspirations which were examined by indirect immunofluorescence with ARAHT; myeloid to erythroid ratios were 3.4–2.4/1, and very little dilution by peripheral blood was present. Individual values for percent of lymphocytes staining in these instances were 8, 19, 20, and 26%; concurrent staining for surface Ig in these samples showed a majority of the remaining cells to be positive—e.g., in the patient with 19% T-cells in marrow lymphocytes, there were 66% surface Ig containing cells.

ponent) and 23 A (for red component) as previously described by others (36, 37). Double staining for Ig employed tetramethylrhodamine isothiocyanate-conjugated anti-IgG and fluorescein-conjugated anti-IgM or anti-IgA. Simultaneous application of these conjugates as well as prior application (15–20 min) of one or the other produced similar or comparable results. When double staining for T- and B-cells was performed, rhodamine-conjugated anti-Ig was generally added simultaneously with or before the ARAHT diluted 1:2, followed by application of fluorescein-conjugated goat antirabbit γ-globulin. Again, reversal of the order of addition of conjugates or ARAHT did not appreciably affect our results.

Specificity of indirect immunofluorescent staining of lymphocytes using ARAHT followed by fluoresceinated goat antirabbit IgG was confirmed by complete inhibition of staining using prior blocking of ARAHT-incubated cells with unconjugated goat antirabbit IgG. In addition, the fluorescein-conjugated goat antirabbit IgG added to lymphocytes alone produced no detectable staining. Absorption of the ARAHT with fresh living human fetal thymocytes (using 10×10^6 cells/ml of ARAHT) markedly reduced subsequent indirect staining. Similar absorptions of ARAHT with living human thoracic-duct lymphocytes (obtained from patients with RA or one subject with chronic glomerulonephritis) likewise produced considerable diminution in indirect fluorescence, however, the thoracic duct cells were not as effective in such absorptions as living human thymocytes.

ARAHT showed striking indirect fluorescence of 96–99% of five different individual fresh samples of living human thymocytes obtained at therapeutic abortion. ARAHT stained 35–50% of fresh splenic lymphocytes prepared from human spleens removed at surgical procedures and 8–26% of lymphocytes obtained after Ficoll separation of fresh human bone marrow secured from normal donors. In addition, ARAHT antisera stained between 95 and 100% of cells collected by thoracic duct drainage of three different patients with RA.[2] Peripheral blood lymphocytes from one adult subject with acquired hypogammaglobulinemia showed 95% reactivity with ARAHT. The specificity of the ARAHT antiserum is shown in Table I. Cells identified as T-cells using ARAHT antiserum and indirect immunofluorescence were living cells as judged by their trypan blue dye exclusion. It was noted that if preparations contained a considerable portion of dead cells, a homogeneous general staining considered nonspecific was observed. No specificity for any human HL-A constellations of antigens or haplotypes was observed when a panel of 20 normal human lymphocytes of varying HL-A type were tested. Peripheral encircling rim or patchy peripheral fluorescence was the characteristic staining pattern noted in many preparations of lymphocytes examined. Characteristic staining patterns using the ARAHT are shown in Fig. 2. It is important to stress that the specificity of the T-cell-specific antiserum appeared to depend on testing conditions insuring living lymphocyte populations.

RESULTS

B-cell identification. The distribution of B-cells among normal controls, and patients with RA, SLE, and tuberculosis as identified by percentages of peripheral blood lymphocytes showing surface γA, γG, and γM is shown in Table II. No significant differences in means for percentages of cells staining for γA, γG, or γM were noted between normals and subjects with RA. Among the patients with SLE, marked variations were noted in the range of values recorded for surface Ig percentages,

[2] Living thoracic duct cells obtained by human thoracic duct drainage were generously provided by Doctors James B. Peter and Carl M. Pearson, UCLA Medical Center.

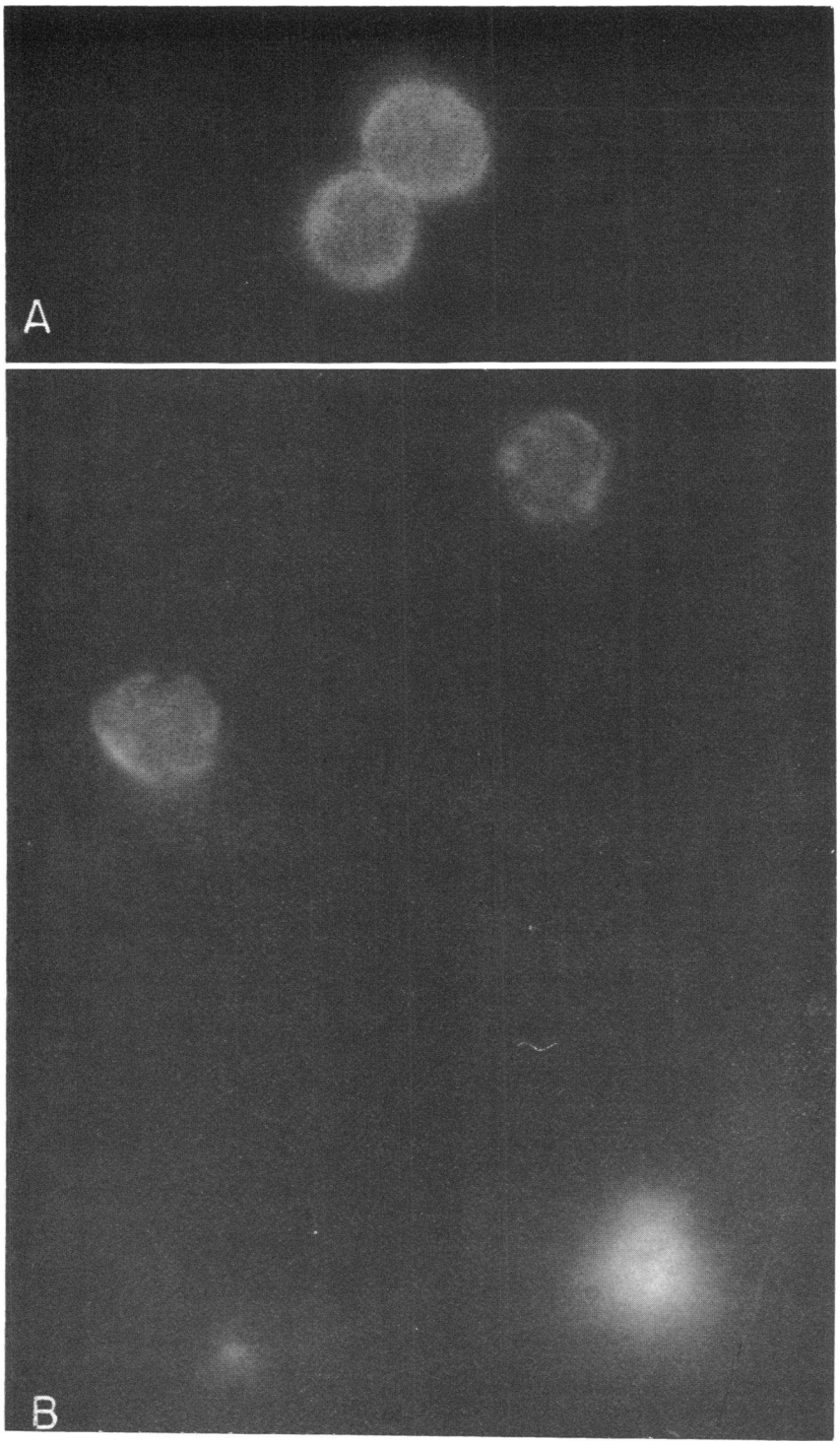

FIGURE 2 Typical staining patterns of living normal human lymphocytes stained with rabbit antihuman fetal thymocyte antiserum absorbed five times with 60×10^6 human CLL cells (ARAHT). On the top (A) is shown the typical complete encircling rim pattern seen in most instances, whereas on the bottom (B) patchy surface fluorescence with the same antiserum is shown. In the lower right hand corner of Fig. 2 (B) a crenated dead cell shows the homogeneous nonspecific pattern seen with dead cells. Specificity of ARAHT was clear-cut only when living cells or cell suspensions were used.

TABLE II

Peripheral Blood Lymphocytes Staining for Surface γA, γG, and γM

	γA	γG	γM
		%	
1. Normal controls			
Sample size	50	57	56
Range	(0–13)	(3–25)	(1–13)
Mean	3.26	11.70	6.64
SD	2.59	4.28	2.79
Median	3	11	6
2. RA			
Sample size	54	59	64
Range	(0–9)	(2–25)	(0–19)
Mean	2.87*	10.42*	5.80*
SD	2.27	5.51	4.49
Median	2	9	4
3. SLE			
Sample size	21	25	24
Range	(0–30)	(2–64)	(1–44)
Mean	3.48*	13.52*	8.00*
SD	6.41	12.75	9.06
Median	2	10	5
4. Active tuberculosis			
Sample size	21	21	21
Range	(0–16)	(5–28)	(0–16)
Mean	3.67*	13.95*	6.95*
SD	4.05	5.41	4.31
Median	3	12	6

* Comparison of these mean values with those of normal controls by t test indicated no significant differences. P values in all instances were >0.05.

reflected in the large standard deviations recorded in Table II. In general, mean B-cell values among patients with tuberculosis were not significantly different from normals.

Normal lymphocyte donors. The relative distributions of B- and T-cells among 35 normal subjects studied concomitantly for surface Ig (B-cells) as well as with the ARAHT antiserum are given in Table III. The sum of percentages of normal cells staining for Ig (B-cells) plus those concurrently recorded as positive with ARAHT (T-cells) ranged from 70 to 119% (average 98.6%) indicating reasonable correlation of the sums of the two methods. Individual data and ranges for percentages of peripheral blood T-cells in the total of 51 normal subjects studied as indicated by the ARAHT method is shown graphically in Fig. 3. The mean value for percent T-cells in normals was 75.3±13.95.

Correlation between H-chain subclass and surface staining for κ- or λ-light chain determinants. As a check on the reliability of adding percentages of cells staining for γG, γA, γM together to obtain a figure for B-cells, patients' lymphocytes were also stained with fluoresceinated specific antibody of anti-κ or anti-λ activity, and sums of cells staining for γG, plus γA plus γM compared with those staining for κ- plus λ-determinants. Reasonably good correlation of these sums was obtained (Table IV) although values for κ- plus λ-staining were consistently slightly lower than figures for γA + γG + γM. No direct conclusion could be drawn from these observations but the slightly lower values for κ plus λ were consistent with the idea that a few B-cells might indeed contain more than one surface immunoglobulin marker.

Studies in patieints with RA. In general, mean values for percentages of B-cells as identified by the percentage of surface Ig containing cells were slightly lower in patients with RA than among the normal controls studied but these individual differences were not significant. (Table II).

When the relative distribution and range of percentages of T-cells were examined among 41 patients with RA (Fig. 3) the range in many patients was quite similar to that which had been recorded in normals, however, there appeared to be a considerable number

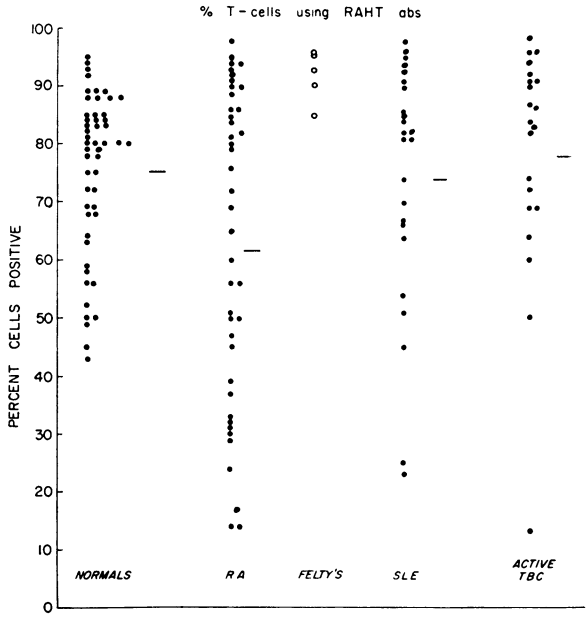

FIGURE 3 This figure shows individual values as well as means for percentages of peripheral blood T-cells identified by indirect immunofluorescence among the various groups of patients studied. The means are not bracketed by 1 SD because a bell-shaped distribution of values was often not encountered. The means and 1 SD for the groups studied were: normals 75.27±13.95%; RA 63.50±26.17%; SLE 74.04±21.26%; and active tuberculosis 77.8±19.73%.

TABLE III

Peripheral Blood Lymphocyte Enumeration as Presumptive T- or B-Cells in 35 Normal Subjects

Subject studied	Lymphocytes bearing surface Ig (B-Cells)			ε-Percent	Lymphocytes staining	Total
	γA	γG	γM	γA+γG+γM	as T-Cells	T+B
		%			%	%
1. W. F.	6	5	10	21	49	70
2. D. A.	7	16	13	36	50	86
3. J. V.	13	15	11	39	56	95
4. K. G.	7	14	6	27	56	83
5. A. M.	3	6	4	13	58	71
6. R. G.	2	10	4	16	63	79
7. B. D.	5	14	8	27	64	91
8. M. S.	1	13	7	21	66	87
9. M. T.	1	10	7	18	68	86
10. W. B.	2	6	8	16	69	85
11. H. B.	3	12	5	20	72	92
12. D. F.	1	9	6	16	75	91
13. R. O.	3	10	9	22	75	97
14. D. L.	6	20	11	37	78	115
15. S. S.	5	11	9	25	78	103
16. M. H.	2	14	10	26	79	105
17. R. G.	9	13	10	32	79	111
18. F. L.	4	15	8	27	80	107
19. J. S.	5	11	6	22	80	102
20. R. B.	1	17	5	23	80	103
21. W. S.	4	6	10	20	80	100
22. J. R.	1	8	2	11	81	92
23. C. F.	6	10	3	19	82	101
24. R. W.	0	11	4	15	83	98
25. S. S.	5	11	9	25	84	95
26. J. R.	5	14	6	25	84	109
27. R. M.	6	19	6	31	84	115
28. K. P.	5	13	9	27	85	112
29. L. T.	4	13	9	26	85	111
30. T. H.	0	9	6	15	85	100
31. C. R.	5	17	7	29	88	117
32. M. L.	1	25	4	30	89	119
33. T. C.	4	5	6	15	89	104
34. K. K.	1	11	7	18	93	111
35. B. H.	1	8	4	13	94	107
						mean 98.6

of patients with very low values 11 patients showing only 14–39% detectable peripheral blood T-cells by the ARAHT fluorescent method. The mean T-cell value within the total group of 41 RA patients studied was 64.5±26.17. In comparing these data with T-cell values recorded in normals the ordinary *t*-test for differences between means was not suitable since the standard deviation for the RA patients was almost double that in the normal group. For that reason the Behrens-Fisher test which does not require that two groups compared show equal standard deviations was used. According to this technique the P value was $0.01 < P < 0.05$. The values for percentages of peripheral blood T-cells among RA patients appeared to cluster in two groups—one within the normal range and a second showing low values. The B- and T-cell data on two groups of 18 patients where simultaneous data were available for T- and B-cell percentages are shown in Table V. Group A (patients 1–18) showed percentages of T-cells from 14–65%; group B (patients 19–36) showed 69–98% T-cells. The two groups were arbitrarily divided in this fashion and an attempt made to determine whether the finding of low total peripheral blood T-cell values correlated in any way with clinical disease activity or therapy. A sum-

TABLE IV

Comparative Percentages of Human Peripheral Blood Lymphocytes Staining for Surface Ig ($\gamma G+\gamma A+\gamma M$) with those Showing Surface Staining for κ- or λ-Determinants

Patients studied	Sum of % cells showing surface Ig (A+G+M)	Staining κ	Staining λ	Sum κ+λ
		%	%	
A. Normals				
1. D. S.	20	12	6	18
2. D. L.	37	21	16	37
3. B. D.	27	17	10	27
4. D. D.	14	9	7	16
5. D. C.	23	18	7	25
6. S. Mc.	35	20	11	31
7. D. R.	36	15	11	26
8. J. S.	22	12	14	26
9. C. R.	29	16	4	20
10. C. F.	19	7	4	11
11. R. Mc.	35	10	14	24
12. B. M.	27	13	5	18
13. D. E.	26	12	6	18
14. R. G.	32	13	11	24
15. T. C.	15	11	6	17
	mean 26.3			mean 22.6

mary of these data are given in Table VI. When low T-cell values in patients with severe rheumatoid activity (3+–4+ clinical grading) were compared with those showing lesser degrees of active disease (1+–2+), a borderline difference was noted by chi-square testing ($P = 0.038$). These data appeared to indicate that low peripheral blood T-cell values tended to be seen in patients severely affected by rheumatoid disease, however, a highly significant correlation was not recorded.

The most interesting finding recorded in the present study was noted among the patients with RA, listed as group A in Table V where the total sum of B-cells as identified by the sum of cells showing direct surface immunoglobulin plus the percentage of T-cells as stained with ARAHT antiserum was quite low (21–77%) accounting for only a fifth to four-fifths of the total peripheral blood-lymphocyte population studied. Examples of this phenomenon are noted in Table V particularly among the first 12 patients listed in group A. Indeed RA patient 2 (R. C.) showed only 21% of peripheral blood lymphocytes that could be accounted for as B- + T-cells using these methods. These results could not be accounted for solely on the basis of steroid therapy which the individual patients were receiving since two of the three lowest total percentages 21% patient 2 (R. C.) and 43% patient 3 (0. L.) were noted in individuals receiving salicylates alone. In addition, the null cells did not correlate with detectable increases of monocytes when Wright's-stained smears of lymphocytes or polystyrene bead additions (34) were carefully monitored in these same patients. It is important to note that this finding of an apparently large population of null cells not accounted for as B- or T-cells by the methods outlined here was not encountered among any normal subject studied.

The findings recorded here were felt to be compatible with the concept that in some patients with RA, a large population of the peripheral blood lymphocytes were somehow different and either as T-cells or B-cells showed diminished or altered reactivity in the test systems used. By contrast the total percentages of B- + T-cells enumerated in normal controls similarly studied ranged from 70 to 119% with many patients showing sums extremely close to 100% (average 98.6%). Clear definition of the precise origin and derivation of the unaccounted for, or null, cells among the RA patients with low total sums of B- and T-cells must await more discriminating techniques than were available in the current study.

Felty's syndrome. All five patients with RA and Felty's syndrome showed relatively low total values for B-cells (range 6–16), mean 9.1% and high percentages of T-cells ranging from 85–96%. These latter data are shown as open circles in Fig. 3.

Systemic lupus erythematosus. In all, 24 individuals were studied for percentage of peripheral blood lymphocytes as T-cells and in 21 individuals, data on T-cells plus B-cells were recorded. These findings are shown graphically in Fig. 3 and also in Table VII. As was noted among the group of patients with RA, in some

TABLE V

Comparative Percentages of RA Patients' Peripheral Blood Lymphocytes Enumerated as Presumptive T- or B-Cells

Patient studied	Lymphocytes bearing surface Ig (B-Cells)			Sum percent	Lymphocytes staining	Total	Type of therapy	Clinical disease activity
	γA	γG	γM	γA+γG+γM	as T-cells	T+B		
		%		%	%	%		
Group A								
1. R. B.	1	15	4	20	14	34	methylpred. 8 mg*	4+
2. R. C.	0	7	0	7	14	21	salic.	4+
3. O. L.	5	16	6	27	16	43	salic.	3+
4. J. L.	5	15	7	27	24	51	salic., gold	2+
5. J. Q.	5	12	7	24	29	53	pred. 10 mg	3+
6. C. F.	0	9	3	12	30	42	salic., gold, pred. 5 mg	3+
7. A. S.	1	10	1	12	31	43	salic., pred. 5 mg	2+
8. V. L.	6	6	8	20	32	52	gold	2+
9. D. M.	6	15	5	26	33	59	salic., gold	3+
10. E. S.	2	7	6	15	37	52	salic.	1+
11. J. S.	1	12	3	16	39	55	salic.	3+
12. S. F.	0	9	2	11	45	56	no therapy	3+
13. W. P.	1	7	7	15	47	62	salic., gold	1+
14. E. L.	1	13	2	16	50	66	salic., pred. 5 mg	2+
15. L. M.	1	13	8	22	50	77	salic.	2+
16. A. M.	5	12	0	17	56	73	salic., pred. 7.5 mg	2+
17. E. L.	2	11	3	16	60	76	salic.	2+
18. T. N.	1	7	4	12	65	77	salic., gold	3+
Group B								
19. M. D.	2	16	8	26	69	95	salic.	2+
20. W. W.	2	9	0	11	72	83	gold, pred. 7.5 mg	2+
21. C. B.	3	11	13	27	75	102	no therapy	4+
22. E. C.	5	20	14	39	79	118	salic.	1+
23. A. R.	6	20	8	34	80	114	salic.	3+
24. M. N.	5	8	2	15	82	97	salic.	2+
25. R. B.	6	14	18	38	84	122	salic.	1+
26. M. A.	5	8	4	17	85	102	salic., indocin, pred. 2.5 mg	1–2+
27. L. P.	1	9	3	13	86	99	salic., gold, pred. 2.5 mg	2+
28. S. S.	8	13	13	34	89	123	salic.	2+
29. M. R.	8	25	15	48	90	138	salic.	0
30. B. P.	5	17	15	37	91	126	salic., indocin	2+
31. G. D.	9	25	17	51	92	143	salic.	1+
32. G. D.	1	13	4	18	93	111	salic., gold	2+
33. M. B.	3	7	3	13	94	107	indocin	2+
34. T. B.	3	4	4	11	94	105	salic.	3+
35. M. S.	3	13	3	19	95	114	salic.	1+
36. C. M.	2	4	1	7	98	105	salic.	1+

* Medications listed indicate therapy at time patient tested; average daily doses of steroid preparations—methylprednisolone or prednisone are indicated.

individuals (three patients) a low total identifiable total percentage of T- + B-cell sum was present.

No correlation could be seen between degree or type of clinical SLE disease activity and the relative percentages of T- or B-cells enumerated. Most of the patients with SLE studied were receiving moderate to high doses of immunosuppressive agents.

The finding of a total of T- + B-cell percent value of 212 in patient 7 (S. L.) in Table VII when cells counted as B-cells plus those staining for ARAHT or T-cells were added stood out as a glaring exception to the general rule. It was clear that at least in this intance some overlap in cell classification must be occurring. The most likely explanation for the extremely

TABLE VI

Clinical Comparison between RA Patients Showing Relatively Low T-Cell Counts with Those Showing Higher T-Cell Values

Patient group	Clinical disease activity				Latex fixation			Steroid therapy	Gold therapy
	4+	3+	2+	1+	>2560	2560	<2560		
A (Pt. 1–18)*	2	7	7	2	4	9	5	6	6
B (Pt. 19–36)*	1	2	8	7	2	8	8	3	3

* Comparison of numbers of patients with severe disease activity (3–4+) vs. moderate to mild activity (1–2+) showed $0.01 > P < 0.038$ by chi-square analysis.

high values recorded for cells containing γA, γG, and γM seemed to be that B-cells in this instance might contain more than one class of surface Ig marker or that some surface bound Ig might represent adsorbed material, such as antigen-antibody complexes, possibly not made by the cell itself. Unfortunately extensive subsequent studies of this particular patient were limited by her leaving the hospital against advice. Double staining technique using fluorescein-conjugated anti-γM and anti-γA and rhodamine-conjugated anti-γG did indeed indicate that half of the B-cells from this particular patient contained at least two classes of surface Ig markers. Clear-cut evidence for this was obtained for γA and γG occurring on the same cell as well as γG and γM. Triple staining was not attempted. Documentation that this could occur was easiest in patients such as S. L. where large percentages of peripheral blood lymphocytes showed surface Ig staining. The degree to which this might be present in normal subjects was much more difficult to assess. In subsequent double-staining studies with normal donors as well as other patients, it was our impression that double staining could indeed occasionally be documented using the criteria originally outlined by Cebra and Goldstein (36).

In addition, double staining using rhodamine-conjugated anti-Ig and fluorescein conjugates for the ARAHT

TABLE VII

Comparative Percentages of Peripheral Blood Lymphocytes as Presumptive T- or B-Cells in Patients with SLE

Patient studied	Lymphocytes bearing surface Ig (B-cells)			ε Percent γA+γG+γM	Lymphocytes staining as T-cells	Total T+B	Type of therapy
	γA	γG	γM				
		%		%	%	%	
1. D. P.	3	12	4	19	15	34	imuran 150 mg; pred. 15 mg*
2. J. S.	2	5	5	12	23	35	salic.
3. P. Mc.	1	14	13	28	45	73	pred. 60 mg
4. V. K.	0	6	1	8	51	58	imuran 100 mg; pred. 60 mg q.o.d.
5. D. B.	2	13	17	32	66	98	pred. 60 mg
6. M. D.	1	4	2	7	70	77	pred. 150 mg q.o.d.
7. S. L.	30	64	44	138	74	212	pred. 60 mg
8. L. W.	0	5	6	11	81	92	pred. 15 mg
9. M. L.	2	11	2	15	81	96	methyl pred. 56 mg q.o.d.
10. L. T.	2	7	10	19	82	101	salic.
11. E. C.	4	22	10	36	83	119	pred. 40 mg
12. G. H.	0	7	3	10	84	94	pred. 40 mg
13. M. E.	2	18	13	33	84	117	IV steroids 1 g+previous low dose steroids (pred. 5 mg)
14. C. O.	1	7	2	10	87	96	pred. 60 mg q.o.d.; imuran 75 mg
15. J. D.	6	22	5	33	90	123	pred. 80 mg
16. M. F.	0	10	5	15	91	106	salic.
17. B. D.	4	7	5	16	93	109	pred. 50 mg
18. L. N.	0	15	6	21	94	115	plaquenil 50 mg
19. D. P.	8	10	15	33	95	128	pred. 40 mg q.o.d.
20. B. N.	1	15	3	19	96	115	none
21. L. O.	3	7	14	24	98	122	pred. 12.5 mg; imuran 50 mg

* Doses of drugs listed represent average daily dose; pred. represents prednisone, salic. represents salicylates.

TABLE VIII

Comparative Percentages of Peripheral Blood Lymphocytes as Presumptive T- or B-Cells in Patients with Active Tuberculosis

Patient studied	Lymphocytes bearing surface Ig (B-cells)			ε Percent	Lymphocytes staining as T-cells	Total
	γA	γG	γM	γA+γG+γM		T+B
		%			%	%
1. L. J.	3	14	12	29	13	42
2. M. S.	1	12	3	16	50	66
3. V. S.*	1	12	5	18	60	78
4. T. L.	3	20	9	32	64	96
5. W. Y.*	16	28	13	57	68	125
6. R. L.*	1	10	7	18	69	87
7. H. S.	6	16	7	29	72	101
8. A. T.	10	19	15	44	74	118
9. J. G.	9	19	15	43	81	124
10. W. M.	0	17	5	22	83	105
11. T. O.	6	12	5	23	84	107
12. P. L.	2	11	0	13	87	100
13. T. M.	1	11	10	22	87	109
14. B. C.	0	5	4	9	90	99
15. B. C.	1	10	11	22	90	111
16. G. M.	5	7	3	15	91	106
17. G. G.	3	9	5	17	92	109
18. M. S.	0	14	1	15	92	107
19. J. K.	1	22	6	29	94	123
20. E. S.	4	14	6	24	96	120
21. P. D.	5	11	4	20	97	117

* Miliary tuberculosis.

T-cell staining was performed in 10 normal subjects. In no instance was significant double staining documented confirming the idea that these methods in use for identifying T-cells and B-cells showed no detectable overlap.

Active tuberculosis. The data obtained on T- and B-cells among the 21 patients with active acid-fast infection are shown in Table VIII. Of great interest was the occurrence of a very low percent of identifiable T-cells in patient L. J., indicating that the latter phenomenon was not a feature peculiar to patients with connective tissue disease. No correlation between degree of disease activity or specific drug therapy and percentage of identifiable peripheral blood T-cells or B-cells was seen, except that the three patients with documented miliary tuberculosis 3 (V. S.), 5 (W. Y.), and 6 (R. L.), Table VIII, showed relatively low peripheral blood T-cell values. More extended serial observations on individual patients are necessary before this question can be completely settled.

DISCUSSION

The studies reported here provide an initial attempt to define peripheral blood and tissue distribution of T-cells and B-cells in patients with the connective tissue diseases such as RA or SLE. The major contribution outlined in the current report relates to the studies of peripheral blood lymphocytes using the rabbit antiserum which after appropriate absorption, appeared to show some degree of specificity for lymphocytes derived from or related to the T-cell system.

The most interesting finding uncovered during the present study lay in the finding that in certain patients with RA and SLE and occasionally in active tuberculous subjects there appeared to be a certain segment of the peripheral blood lymphocytes which were not readily identifiable by the methods used either as T-cells or B-cells. The exact identity of these null cells presents an intriguing question. Particularly in many instances of active RA these cells constituted as many as 48–79% of peripheral blood lymphocytes. In patients with extremely active RA a tendency for low levels of identifiable T-cells was noted, however, no direct correlations were possible between low T-cell percentages and clinical disease activity in patients with SLE. Three patients with miliary tuberculosis showed T-cell percentages at the lower range of values when compared with 18 subjects with other forms of active acid-fast infection. Currently studies are underway using the recently described sheep-cell rosette method for human

T-cell identification (38–40) to attempt to characterize the origin or type of null cell encountered in our studies to date. Preliminary data essentially using the technique described by Fröland (39) indicate that in normal subjects our T-cell direct ARAHT antiserum generally identifies considerably more (10–40%) peripheral blood lymphocytes than does the sheep-cell rosette technique. Further comparative work along these lines is certainly indicated.

The presence of either low or high percentages of B-cells bearing detectable surface Ig in the face of severe clinical lupus activity in several patients suggests that serial studies of B-cells and their turnover may relate such findings to changes in clinical activity. As mentioned above, the finding in some patients with SLE of lymphocytes bearing more than one detectable surface localized class of Ig does not imply that such Ig was all a product of these cells. Adherent antigen-antibody complexes or Ig adsorbed to receptors for antigen-antibody complexes (41, 42) may be involved in such instances. Further studies of this phenomenon are cetrainly needed. Further definition of possible functional subgroups of B- or T-cells is clearly necessary.

A word of restraint is certainly indicated in an evaluation of our data and some of the general approaches outlined here, particularly with respect to whether or not CLL cells can be regarded or used as pure populations of B-cells as in the absorption experiments illustrated in Fig. 1. Although the CLL cells used contained relatively high proportions of cells bearing surface Ig (80–95%), it is conceivable that such cells may contain cell-surface antigenic deletions related to the leukemic process itself. Other workers have encountered some degree of anomalous behavior when leukemic cells have been studied with standard anti-HL-A typing antisera (43). Thus CLL cells may not be the ideal source of a B-cell population with which to absorb antihuman thymocyte antisera. Current studies are under way to define preparative methods by which B-cells may be harvested from normal human donors. The techniques recently described by Abdou (44) using columns of anti-Ig-coated beads may provide a practical solution to this problem.

Regardless of the theortic objections to the use of CLL cells in absorption of antithymocyte antisera, the results reported here would appear to indicate some degree of T-cell specificity obtained with the absorbed rabbit antisera. The virtual 100% reactivity for fetal thymocytes, 42% activity against living human splenic lymphocytes, and 18% staining of B-cells would argue in favor of reactivity for antigens similar to the θ-system in mice. In addition, the absence of any evidence for double staining when rhodamine conjugates for anti-Ig and fluorescein-conjugated anti-T-cell markers were examined concurrently is a strong point in favor of the specificity of the approach outlined here.

For years investigators and clinicians alike have been enthralled by the myriad of autoantibodies demonstrable in the sera of patients with SLE and RA. The purpose of this report would be well served if now more attention were directed at the possible effector cells—lymphocytes—in these disorders. Clinical examples of the potential importance of circulating lymphocytes have been recently reported in which a remission of severe active RA was produced through long-term lymphocyte depletion via thoracic duct drainage (45, 46). A better definition and more precise insight into T-cell and B-cell kinetics and behavior may eventually provide us with considerable basic understanding of the connective tissue disease.

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