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Human blood L lymphocytes in patients with active systemic lupus erythematosus, rheumatoid arthritis and scleroderma: a comparison with T and B cells

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SUMMARY

Human blood lymphocytes with high affinity Fc receptors for IgG will bind small aggregates of this immunoglobulin at 4°C. These cells have been named L lymphocytes because of membrane-*labile* IgG determinants. L cells possess a profile of surface markers and functional characteristics which differ from T and B cells. Immunofluorescence methods have been employed to quantify L lymphocytes in subjects with connective tissue diseases and certain infections, and these values have been compared with those for T and B cells. The mean values of L lymphocytes in groups of patients with systemic lupus erythematosus, rheumatoid arthritis and scleroderma ranged between 14 and 18%; values similar to normals. Groups with acute pneumonia and tuberculosis, however, had significantly increased percentages of L lymphocytes. The absolute number of L cells was decreased in subjects with connective tissue diseases, as was the number of T and B cells. L lymphocytes in those with infections were not significantly decreased. Only L lymphocytes were depleted by immobilized antigen–antibody complexes, another characteristic which distinguishes them from T and B cells.

INTRODUCTION

Human blood lymphocytes have been divided into two major populations, T and B cells, on the basis of animal experiments (Greaves, Owen & Raff, 1973), immunodeficiency disorders (Good, 1971) and cell markers (Warner, 1974). In addition, evidence to suggest a third lymphocyte population has appeared (Frøland & Natvig, 1973; Horwitz & Garrett, 1977). T and B cells have been quantified in subjects with connective tissue diseases, but the results have been controversial. T cells are generally quantified by the E-rosette method and B cells have been quantified by immunoglobulin and complement receptors.

In systemic lupus erythematosus (SLE), percentages of E rosette-forming T cells have been reported as normal (Winchester *et al.*, 1974) or decreased (Williams *et al.*, 1973; Messner, Lindstrom & Williams, 1973; Yu *et al.*, 1974; Sheinberg & Cathcart, 1974; West *et al.*, 1976). In SLE, B cells have been reported as normal (Williams *et al.*, 1973; Sheldon, Papamichail & Holborow, 1974; Yu *et al.*, 1974; Mellbye *et al.*, 1973) or decreased (Sheinberg & Cathcart, 1974). Messner *et al.* (1973) reported increased Ig-positive cells, but decreased C3 receptor lymphocytes.

In rheumatoid arthritis (RA), except for one report of decreased T cells, several groups have found normal values (Sheldon *et al.*, 1974; Winchester *et al.*, 1974; Williams *et al.*, 1973; Yu *et al.*, 1974; Keith & Currey, 1973). B cells in this disease have been considered to be increased (Papamichail, Brown & Holborow, 1971; Gergely *et al.*, 1973) or normal (Williams *et al.*, 1973; Yu *et al.*, 1974; Mellbye *et al.*, 1973; Frøland, Natvig & Husby, 1973). In addition, Williams *et al.* (1973) and Scheinberg & Cathcart (1974) have reported large numbers of cells with neither T- nor B-cell markers (null cells).

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These controversial results may be explained by three major factors: (a) differences in patient populations, (b) differences in sensitivity of methodology and (c) failure to account for 'third population' lymphocytes. Values for T cells obtained by E rosettes reflect the sensitivity of the technique. Moreover, it has been found that non-T cells will form E rosettes under certain conditions (Weiner, Bianco & Nussenzweig, 1973). Secondly, Winchester *et al.* (1974) found that many Ig-positive cells in these diseases were not B lymphocytes, but were other cell species that had bound anti-lymphocyte antibodies. Thirdly, Lobo, Westervelt & Horwitz (1975) revealed that B cells in the blood of normal subjects had been greatly overestimated. Two separate populations of lymphocytes bearing easily detectable surface Ig were described; one with surface-stable Ig determinants (B cells) and another population that Horwitz & Lobo (1975) named L lymphocytes because of membrane-*labile* IgG determinants. These cells were identical with the 'third population' that Frøland & Natvig (1973) had described by an Fc receptor–rosetting method (Lobo & Horwitz, 1976).

'L' cells generally comprise 10 to 20% of blood lymphocytes* and are identified by a highly avid Fc receptor that binds small aggregates of IgG in serum at 4°C and releases them at 37°C (Horwitz & Lobo, 1975). L cells do not form rosettes with sheep red blood cells (SRBC). They also lack membrane-incorporated Ig and do not possess a C3 receptor capable of forming rosettes with erythrocytes sensitized with IgM antibody and mouse complement (Horwitz & Lobo, 1975; Ehlenberger *et al.* 1976). B cells (Dickler & Kunkel, 1972) and a subset of T cells (Samarut, Brochier & Revillard, 1976; Moretta *et al.*, 1976) also possess Fc receptors for IgG. The avidity or density of receptors on these cells, however, is less than the Fc receptors on L lymphocytes. Only L lymphocytes bind human erythrocytes sensitized with Ripley anti-Rh IgG (Frøland & Natvig, 1973; Lobo & Horwitz, 1976; Kurnick & Grey, 1975).

The purpose of the present experiments has been to quantify L lymphocytes in subjects with connective tissue diseases and to compare these values to those of subjects with infectious diseases. In addition, T and B cells have also been quantified in all groups. Appropriate precautions have been taken to distinguish the two populations of immunoglobulin-bearing lymphocyte populations from T cells, which have fixed anti-lymphocyte antibodies on their surface membrane.

MATERIALS AND METHODS

Subjects. Three experimental groups included twenty-two subjects with SLE, thirty-six subjects with RA and ten with scleroderma. All of these subjects were hospitalized at the University of Virginia Medical Center. Three control groups included seventeen subjects with acute infections, twelve with tuberculosis and thirty-five normals. Subjects with both groups ofinfections were hospitalized. The age and sex of each group studied is given in Table 1. Patients with SLE had objective clinical involvement of at least two of the organ systems commonly affected by this disease, and had antinuclear antibodies. Seventeen of these subjects were untreated and five others were receiving 10 mg or less of prednisone. None were being treated with cytotoxic agents. All patients with rheumatoid arthritis had definite disease by the American Rheumatism Association Criteria (Ropes *et al.*, 1968). All subjects were receiving aspirin or other non-steroidal anti-inflammatory agents.

TABLE 1. Clinical data of patients with connective tissue diseases and infections

Patients	Male	Female	Age (years)	WBC	Small lymphocytes (%)
Normals	17	18	31 (23–48)	6271±240	32·5±1·6
Connective tissue diseases					
SLE	3	19	34 (17–58)	5459±382†	18·6±1·3‡
RA	9	27	41 (20–60)	7658±453†	19·1±1·3‡
Scleroderma	7	3	49 (35–54)	7470±1211	18·3±3·3‡
Infections					
Acute infections	10	7	35 (20–53)	9659±1088‡	13·9±1·8‡
Tuberculosis	3	9	36 (18–49)	8392±874	22·0±2·2

Values indicated equal the mean ±1 s.e.; WBC, white blood count.

† $P \leqslant 0{\cdot}05$; ‡ $P \leqslant 0{\cdot}001$; Student's *t*-test.

* See note added in proof.

Ten were receiving 10 mg or less of prednisone a day. None were receiving cytotoxic drugs. The subjects with scleroderma had severe far-advanced disease. Fourteen of the subjects with acute infections had pneumonia, two had pyelonephritis and one had acute sepsis. Patients with tuberculosis were admitted to the Blue Ridge Tuberculosis Sanitorium because of active pulmonary disease. All studies were conducted within the first 5 days of admission to the hospital.

Separation of lymphocytes from peripheral blood. 20 ml of blood was drawn into a plastic syringe and diluted 1:1 with sterile physiological saline containing 200 u of preservative-free heparin (Grand Island Biological Co., Grand Island, N.Y.). The blood was layered onto Ficoll–Hypaque (LSM, Litton Bionetics, Kensington, Maryland) and spun at 400 *g* at 20 C for 35 min. The interface was carefully aspirated and diluted 1:1 with minimal essential medium (MEM; Grand Island), centrifuged and washed once. The cells were resuspended in MEM with 5% foetal calf serum (Grand Island) (MEM–FCS) to which latex particles (Bacto-Latex, 0·81 μm, Difco Laboratories, Detroit, Michigan) were added and incubated at 37°C in 5% CO_2 for 1 hr. The cells were washed three times with warmed MEM–FCS.

Quantification of B cells. 50 μl of polyvalent goat anti-human immunoglobulin (Hyland Division, Travenol Laboratories, Costa Mesa, California) diluted 1:5 were added to 10^6 mononuclear cells and incubated at 4°C for 30 min. The cells were washed three times in cold MEM–FCS containing 0·02% sodium azide. Wet mounts were prepared and a minimum of 200 cells was counted immediately using a Leitz Ortholux microscope equipped with a ploem illuminator. We have compared these values with cells stained with a fluorescein-conjugated $F(ab')_2$ polyvalent anti-human serum (Capell Laboratories, Downingtown, Pennsylvania). The values obtained with the pepsin-digested conjugate were identical to the whole antiserum, provided the cells are pre-treated at 37°C before staining.

Quantification of L lymphocytes. 50 μl of normal human serum (as a source of IgG) were added to 10^6 cells at 4°C for 30 min. The cells were washed three times, stained as above and counted. This procedure indicates the total number of Ig-positive cells. The number of L lymphocytes is calculated by subtracting B cells from the total number (Fig. 1).

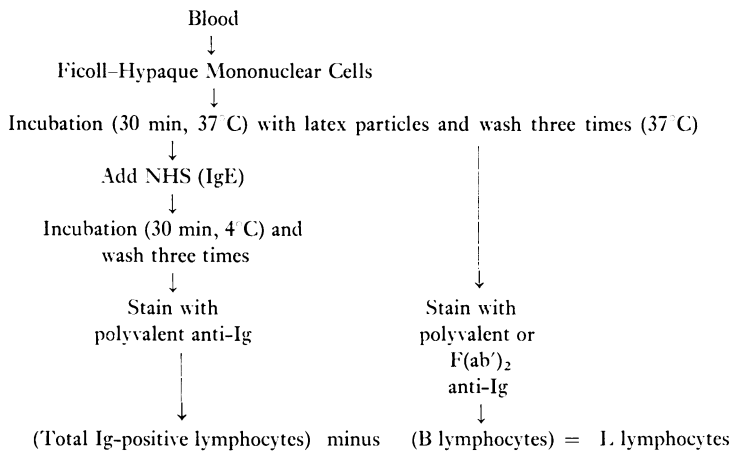

FIG. 1. Description of method for distinguishing L from B lymphocytes.

Detection of autoreactive anti-lymphocyte antibodies. 50 μl of autologous serum was added to 10^6 cells, incubated at 4 C as above, washed and then stained. The serum was considered to have anti-lymphocyte antibodies if the number of lymphocytes stained by autologous serum was increased by at least 1·5 times the value observed when the cells were incubated in normal human serum. The frequency of anti-lymphocyte antibodies determined by this method was equivalent to results by a microcytotoxicity method (Horwitz, Garrett & Craig, 1977).

Quantification of T cells. Sheep cells in Alsever's solution were obtained weekly and washed three times in MEM. 0·2 ml of a 2% sheep cell suspension was added to 10^6 lymphocytes and 0·2% of foetal calf serum (previously absorbed with SRBC). The ratio of SRBC to lymphocytes was 60–75:1. The mixture was incubated at 37°C for 15 min, spun at 200 *g* for 5 min and incubated at 4°C overnight. Cells with three or more SRBC were considered to be positive.

Detection of other surface receptors. Lymphocytes with receptors for mouse complement (EAC) were detected by sensitizing sheep erythrocytes with IgM antibodies (Cordis laboratories, Miami, Florida) and mouse serum by methods described previously (Horwitz & Lobo, 1975). Procedures for identification of lymphocytes forming EA rosettes with human erythrocytes sensitized with Ripley IgG antibodies have also been described (Lobo & Horwitz, 1976).

Depletion of Fc receptor cells. Fc receptor cells can be removed from other lymphocytes by allowing them to adhere to immobilized immune complexes (Alexander & Henkart, 1976). 2 ml of keyhole limpet haemocyanin (KLH; Calbiochem, San Diego, California; 0·5 mg/ml) were incubated on 60 mm plastic petri dishes at 37°C for 30 min and removed. Then 2 ml of a 1:20 dilution of hyper-immune rabbit antiserum or normal rabbit serum was also incubated at 37°C for 30 min. After the plates were washed, 8×10^6 mononuclear cells were suspended in 4 ml of medium and incubated at room temperature for 45 min. The dishes were then shaken and the non-adherent cells were harvested.

RESULTS

The mean number of L lymphocytes in normals was 15% (Table 2). Similar values were observed in groups with SLE, RA and scleroderma. In control groups with infections, however, increased percentages of L lymphocytes were observed. Values for subjects with acute infections and tuberculosis were 23 and 21% respectively, and each was significantly different from the other groups ($P<0{\cdot}05$, Student's t-test).

The mean value of B lymphocytes in normals was 9·4%. Similar values were found in subjects with the three connective tissue diseases and both groups of infections. The mean number of T lymphocytes in normals was 73%. Similar values were found in SLE, the other two connective tissue diseases and those with acute infections. The mean proportion in the group with tuberculosis was 57%, a value that was significantly decreased.

Non-identifiable or 'null' cells were infrequent. In the last sixty consecutive subjects studied, at least 97% of the total number of lymphocytes were identified by either the E-rosette or immunofluorescence method.

TABLE 2. Percentage of L, B and T lymphocytes in patients with connective tissue diseases and infections

Patients	L lymphocytes	B lymphocytes	T lymphocytes
Normal ($n = 35$)	15·3±1·1	9·4±0·7	72·5±1·6
Connective tissue diseases			
SLE ($n = 22$)	16·7±2·4	8·9±1·1	74·9±1·7
RA ($n = 36$)	17·8±1·8	8·7±0·9	69·6±2·1
Scleroderma ($n = 10$)	14·4±1·9	9·1±0·9	67·4±3·1
Infections			
Acute infections ($n = 17$)	22·8±2·8*	9·1±1·3	67·8±3·6
Tuberculosis ($n = 12$)	21·1±3·8*	11·0±2·5	57·2±6·1†

Values indicated equal the mean ±1 s.e.

* $P\leqslant 0{\cdot}05$; † $P\leqslant 0{\cdot}001$; Student's t-test.

The frequency distribution of L lymphocytes in all groups is shown in Fig. 2. 75% of the normal group had between 200 and 500 L lymphocytes per mm^3. Decreased numbers of L lymphocytes were found in the three connective tissue diseases. 75% of subjects with SLE, 60% of subjects with rheumatoid arthritis and 70% of subjects with scleroderma had fewer than 200 L lymphocytes per mm^3. In contrast, only 40% of subjects with acute infections and less than 10% of those with tuberculosis had fewer than 200 L lymphocytes per mm^3.

All three groups with connective tissue disease and those with acute infections were lymphopenic (Table 3, Fig. 3). The absolute numbers of L, B and T lymphocytes were significantly decreased in SLE, RA and scleroderma. A more selective lymphopenia was found in patients with acute infections. The mean value of L lymphocytes was not significantly decreased (Table 3). The mean value of L lymphocytes in the group with tuberculosis was slightly, but not significantly, higher than in the normal group (371 cells per mm^3 and 305 cells per mm^3, respectively). The proportional relationship of L, B and T cells in all groups studied is illustrated in Fig. 3.

The non-selective lymphopenia found in subjects with connective tissue diseases could not be explained by autoreactive anti-lymphocyte antibodies. These antibodies were common in serum from subjects with SLE, less frequent in RA and unusual in scleroderma. All these groups, however, were lymphopenic. Moreover, seven out of twenty-two subjects with SLE had greater than 300 L lymphocytes per mm^3 and five of these subjects had autoreactive anti-lymphocyte antibodies.

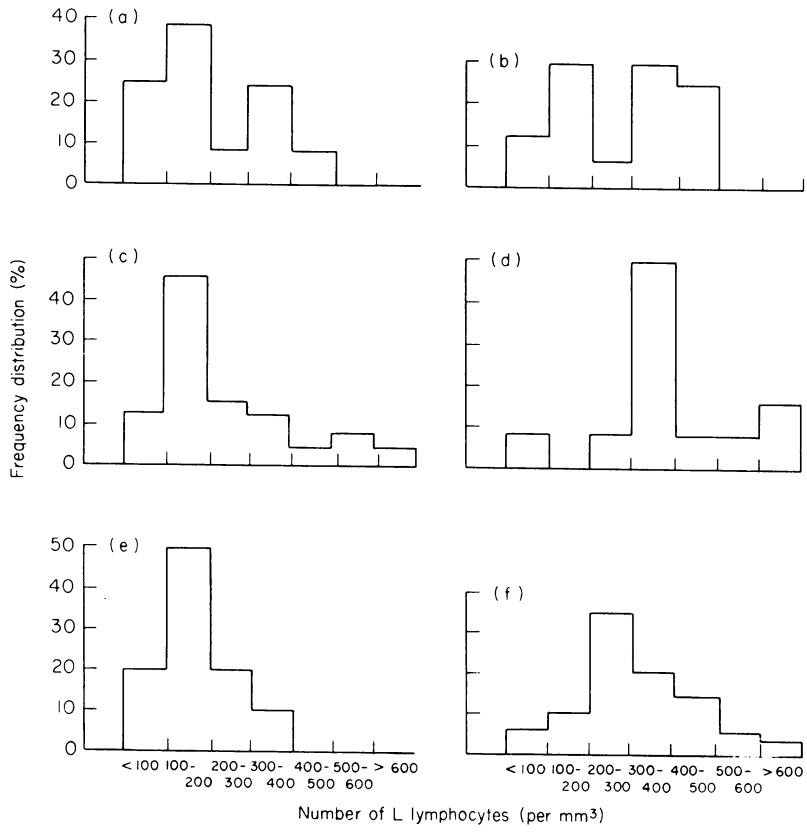

FIG. 2. Frequency distributions of blood L lymphocytes in subjects with connective tissue diseases, infections and normals. (a) SLE, (b) acute infections, (c) RA, (d) tuberculosis, (e) scleroderma, and (f) normal.

TABLE 3. Absolute number of L, B, and T lymphocytes in patients with connective tissue diseases and infections

Patients	L lymphocytes	B lymphocytes	T lymphocytes	Total small lymphocytes
Normals (n = 28)	305±25	206±21	1403±95	2032±103
Connective tissue diseases				
SLE (n = 22)	191±35*	93±15*	753±83*	998±98*
RA (n = 36)	230±26†	134±21†	938±85*	1399±111†
Scleroderma (n = 10)	162±25†	109±20†	782±137*	1166±189*
Infections				
Acute infections (n = 17)	272±41	101±19†	835±155*	1197±161*
Tuberculosis (n = 12)	371±64	186±42	954±138*	1767±214

Values indicated equal the mean ±1 s.e.

* $P \leq 0{\cdot}001$; † $P \leq 0{\cdot}05$; Student's t-test.

In SLE, there was no correlation between the number of L lymphocytes found and the serum complement levels, anti-DNA antibodies or the erythrocyte sedimentation rate. The values of L lymphocytes in rheumatoid arthritis did not appear to correlate with the activity of synovitis.

As already stated, previous studies in normal subjects had revealed that L lymphocytes did not have

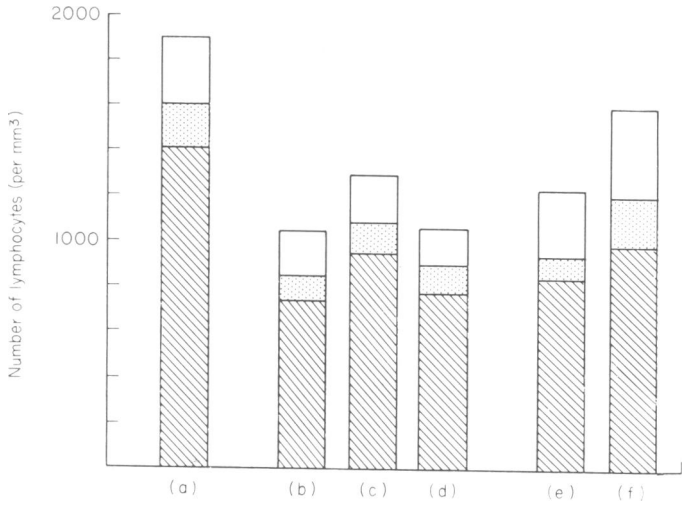

FIG. 3. The proportional relationship of L, T and B lymphocytes in (a) normals, (b) SLE, (c) RA, (d) progressive systemic sclerosis, (e) pneumonia and (f) tuberculosis. (□) L lymphocytes, (▨) T lymphocytes, (▩) B lymphocytes.

receptors for mouse complement and only L cells formed Ripley EA rosettes. Additional studies were performed on lymphocytes from patients with RA to learn whether L lymphocytes from these patients had a similar profile of surface markers. Percentages of T, B and L lymphocytes were determined, as well as lymphocytes forming EAC and EA rosettes. Fc receptor cells were then depleted by allowing them to adhere to soluble immune complexes immobilized to plastic surfaces. As shown in Table 4, this procedure did not affect the number of lymphocytes with membrane-incorporated Ig or EAC rosette-forming cells. E rosette-forming cells increased by 10%. In sharp contrast, cells with surface-labile IgG determinants and with the capacity to form EA rosettes were greatly depleted. These studies confirm previous observations in normal subjects that T and B cells do not possess high-avidity Fc receptors and that L lymphocytes lack receptors for mouse complement.

DISCUSSION

The procedure described in this report enables the investigator to avoid two important pitfalls which have resulted in the over-estimation of human B lymphocytes. Previously, lymphocytes that had bound

TABLE 4. Depletion of Fc receptor lymphocytes by immobilized antigen–antibody complexes*

Patients	L lymphocytes		B lymphocytes		T lymphocytes		EAC		EA	
	Before	After	Before	After	Before	After	Before	After	Before	After
Sha.	7	2	10	7	79	84	8	8	7	0·5
Kin.	10	1	11	9	74	90	8	9	11	1
Car.	12	0	8	7	78	n.d.	8	8	11	0
Tho.	10	0	8	9	77	87	9	11	10	0·5
Mean	9·8	0·8	9·3	8·0	77·0	87·0	8·3	9·0	9·8	0·5

* Immobilized KLH–anti-KLH immune complexes were prepared by incubating KLH (0·5 mg/ml) on plastic petri dishes at 37°C for 20 min, washing and incubating hyper-immune rabbit anti-KLH (1:20) at 37°C for 20 min and washing. Cell suspensions were added and incubated at room temperature for 45 min and the non-adherent lymphocytes were harvested. n.d.=Not done. All results in percentages.

anti-lymphocyte antibodies or that had fixed IgG in normal human serum by Fc receptors had been mis-labelled as B lymphocytes. By pre-treating lymphocytes at 37°C, extrinsic immunoglobulin is eluted from the cell membrane and only B lymphocytes are stained by a polyvalent antiserum.

Winchester *et al.* (1974) employed $F(ab')_2$ anti-Ig to distinguish between B lymphocytes with IgM and IgD receptors from other lymphocytes which lack these determinants but which bind complexed IgG by Fc receptors (our definition of L lymphocytes). In the present experiments, we found that after the cells were pretreated at 37°C, the values for cells stained with whole antiserum and pepsin-digested antiserum were identical.

Evidence is accumulating from studies of cell surface markers, histochemical staining properties and functional analysis of purified cells that L lymphocytes comprise a third separate lymphocyte population, although this issue is not settled. As already stated, the profile of surface markers on L cells is unlike that of T and B cells. The present studies confirmed previous findings that only L lymphocytes possess high-affinity Fc receptors (Lobo & Horwitz, 1976). Ag–Ab complexes immobilized to plastic surfaces depleted L cells, but not T and B cells. These observations do not conflict with the previous observations that B cells and a subset of T cells possess Fc receptors, but indicate receptors of lower affinity or density on these cells.

L lymphocytes from normals lack receptors for mouse complement and these studies revealed a similar situation in subjects with RA. The method we have employed detects mainly C3d receptors. The possibility that L lymphocytes possess weak C3b receptors detectable with other methods or sources of complement has not been excluded.

Besides differences in surface markers, histochemical differences between L lymphocytes and T cells have become apparent. Studies from this laboratory have revealed that T cells characteristically contain granules of α-napthyl esterase close to the plasma membrane. L lymphocytes are non-specific esterase-negative (Horwitz & Garrett, 1977).

The most important evidence that L lymphocytes comprise a unique population has come from functional studies. Horwitz & Garrett (1977) have isolated T (E+, EA−, EAC−), B (E−, EA−, EAC+) and L (E−, EA+, EAC−) lymphocytes by rosetting techniques, and have extensively examined the properties of each population. Unlike T cells, L lymphocytes were unable to proliferate in response to polyclonal mitogens, soluble antigens or histocompatibility antigens. Neither were L lymphocytes a subset of B cells. Unlike the 'null' cells of Chess *et al.* (1974), they did not spontaneously develop surface-stable Ig markers *in vitro*. Neither could pokeweed mitogen induce L cells to undergo B-cell differentiation. Moreover, L lymphocytes were poor stimulators in mixed lymphocyte cultures. This characteristic also distinguishes them from B cells, which are effective stimulators (Lohrman, Novikovs & Graw, 1974). Although L lymphocytes morphologically resemble lymphocytes, there is no evidence to date that these cells possess antigen receptors, the characteristic property of both T and B cells.

L lymphocytes are potent mediators of antibody-dependent lymphocyte cytotoxicity (ADCC). Perlmann (1976) has functionally defined lymphocytes with this capacity as K cells, but it should be emphasized that the two terms are not synonomous. K cells are defined strictly by ADCC. They comprise 5% of total lymphocytes when quantified by a plaque assay, and this number includes a subset of T cells (Perlmann & Wahlin, 1976). Activated T cells also have K-cell activity (Saal *et al.*, 1977). L cells, in contrast, are enumerated by surface markers. They are more numerous and in addition to K-cell activity they have 'natural killer' activity as well (Deteix, Horwitz & Allison, unpublished observation). Moreover, the studies of Ryan & Henkart (1976) in mice suggest that L cells activated by immobilized immune complexes suppress lymphocyte mitogenesis.

The percentage of L lymphocytes was increased in groups with pneumonia and tuberculosis. Niklasson & Williams (1974) have recently reported decreased T cells and elevated percentages of 'B' cells in acute infections. These workers reported, however, that some patients with high numbers of Ig-bearing cells did not have a corresponding increase in complement-receptor lymphocytes. This apparently paradoxical finding is probably best explained by the recent observation that B lymphocytes, but not L lymphocytes, have receptors for erythrocytes sensitized with 19S antibody and mouse complement. It is likely that Niklasson & Williams observed elevated numbers of L lymphocytes and normal numbers of B cells.

Our observations of a normal percentage of T cells in SLE is in agreement with Winchester *et al.* (1974), but conflicts with several groups who have reported decreased values. Since many of the workers who had reported low T-cell values did not pre-treat the lymphocytes at 37°C, it is probable that humoral factors may have interfered with the E-rosette formation. The present finding of normal percentages of B cells in SLE is consistent with other reports. In addition, the finding of normal percentages of T and B cells in subjects with RA is also in agreement with other reports cited in the introduction.

The reason for the L lymphopenia in connective tissue diseases but not in the infections is unknown. One possibility is that cell-bound anti-lymphocyte antibodies may have led to the elimination of these cells *in vivo*. Although a correlation between anti-lymphocyte antibodies, lymphopenia and disease activity had been reported in SLE (Winfield *et al.*, 1975; Utsinger, 1976), additional factors must be postulated. In this study, some subjects with normal numbers of L lymphocytes had autoreactive anti-lymphocyte antibodies. Although most subjects with RA and scleroderma did not have autoreactive anti-lymphocyte antibodies, they were nonetheless lymphopenic. Decreased L lymphocytes may be another manifestation of impaired cell-mediated immunity found in SLE and other rheumatic diseases (Horwitz & Cousar, 1975).

Note added in proof

Studies conducted since this manuscript was submitted have revealed a heterogeneity of L cells. Since the site of origin of these cells may also be heterogeneous, it is appropriate to place quotation marks around our term for high-avidity Fc receptor cells and to call them 'L' cells. Two major subsets, one with low density Ia markers and the other with a myeloid surface antigen, have been described (Niaudet *et al.*, unpublished observations).

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