Combined Studies of Complement Receptor and Surface Immunoglobulin-Bearing Cells and Sheep Erythrocyte Rosette-Forming Cells in Normal and Leukemic Human Lymphocytes

GORDON D. ROSS, ENRIQUE M. RABELLINO, MARGARET J. POLLEY, and HOWARD M. GREY

From the National Jewish Hospital and Research Center, Denver, Colorado 80206 and the Scripps Clinic and Research Foundation, La Jolla, California 92037

ABSTRACT Human lymphocytes from normal peripheral blood, thymus, spleen, thoracic duct, and peripheral lymphocytes from patients with chronic lymphatic leukemia were studied for complement receptor sites (CRL), surface immunoglobulin (SIg), and for the ability to form rosettes with sheep erythrocytes (TRFC). The two B cell markers (CRL and SIg) were found to be in overlapping, but not totally identical populations, whereas cells that were able to form rosettes were found in a totally unrelated population of lymphocytes; TRFC is therefore probably a reliable marker for T cells. In peripheral blood 24% of lymphocytes had SIg, but only half of these were also CRL. Almost all of the non-SIg peripheral blood lymphocytes were TRFC. In the spleen and thoracic duct only a few lymphocytes were observed that had SIg and were not CRL. On the other hand, in two of three spleens studied 10–20% of cells were CRL that did not have SIg. In the thoracic duct all non-CRL, non-SIg cells were TRFC. In chronic lymphatic leukemia three findings were made: (*a*) The presence or absence of CRL was independent of the presence or absence of SIg so that in individuals whose cells were non-SIg, CRL were usually plentiful. (*b*) Leukemic cells were essentially negative for TRFC. (*c*) Leukemic cells reacted poorly with human C3 compared to mouse C3, EACmo detecting up to 20-fold more CRL than EAChu. This latter finding was in sharp contrast to normal CRL that reacted somewhat preferentially with EAChu. These data suggest that altered surface Ig receptors and complement receptors are present in chronic lymphatic leukemic cells. Since the cells obtained from all leukemic patients tested in this study had either the complement receptor or surface immunoglobulin in a high percentage of their cells and were essentially negative for TRFC, it is strongly suggested that leukemic lymphocytes are of B cell origin. The finding of lymphocytes with only one of the two B cell markers suggests that these markers are not uniformly present on all B cells and that depending on the source, one or the other may be deficient.

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INTRODUCTION

Lymphocytes active in the immune response are derived from either the bone marrow (B cells) [1] or from

[1] *Abbreviations used in this paper:* B cells, lymphocytes derived from bone marrow; BSA, bovine serum albumin; CLL, chronic lymphatic leukemia; CRL, complement receptor lymphocytes; EA_{19S}, sheep erythrocytes sensitized with 19S antibody; EAC, erythrocyte-antibody-complement complexes; EDTA, (ethylenedinitrilo)-tetraacetic acid; FCS, fetal calf serum; gp, guinea pig; GVB, gelatin veronal buffer with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (11); HBSS, Hanks' balanced salt solution; $HBSS^=$, Ca,Mg-free HBSS; hu, human; mo, mouse; SIg, surface immunoglobulin; T cell, lymphocytes derived from thymus; TDL, thoracic duct lymphocytes; TRFC, thymus-derived rosette-forming cells; also, 0.0027 M EDTA-$HBSS^=$ was made by addition of 3.2 ml of a 0.087 M EDTA pH 7.2 (isotonic) stock solution to 100 ml of $HBSS^=$.

the thymus (T cells). Since each of these two types of lymphocytes has both independent and cooperative roles in the immune response, it is essential to be able to identify them easily in order to understand their overall function. The majority of B cells have easily detectable membrane-bound immunoglobulin on their surface, while T cells do not (1–3). Most B cells also have a receptor for complement and are termed complement receptor lymphocytes or CRL (4, 5). While studies of each of these two B cell markers have shown that neither is found on T cells, there is some doubt as to whether all B cells have both markers. In the mouse, T cells have the theta antigen and B cells do not. Human T cells can be identified by their lack of B cell markers and by their ability to form rosettes with sheep erythrocytes (6–8).

The purpose of the present study was threefold: (*a*) to try to determine whether surface immunoglobulin (SIg) and the complement receptor were both present on the same lymphocytes in normal individuals, (*b*) to study the ability of human lymphocytes to form rosettes with sheep erythrocytes and determine whether only T cells have this property, and (*c*) to study lymphocytes from patients with chronic lymphatic leukemia (CLL) in order to determine whether these cells more closely resembled B or T cells. Special emphasis was placed on examining those leukemia patients whose cells did not have immunoglobulin detectable by immunofluorescence.

METHODS

Lymphocytes. Blood from normal adults and patients with a confirmed diagnosis of CLL was collected in heparin. Among the CLL patients, lymphocyte counts ranged from 25,000 to usually greater than 100,000 per mm^3, and therefore, presumably the vast majority of the lymphocytes were leukemic. Thymus and spleen cells were obtained from autopsies of individuals known to have no gross immunological defect. Thoracic duct lymphocytes (TDL) were a generous gift from Dr. Joan Stratton and Dr. James B. Peter, and were obtained by continuous drainage of thoracic duct lymph from patients with severe rheumatoid arthritis and other collagen vascular diseases.

Separation of lymphocytes. Heparinized blood was diluted 1:2 with 3% dextran T-250 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 0.0027 M EDTA-HBSS$^=$ (see abbreviation list) and allowed to sediment for 40 min at room temperature. The leukocyte-rich supernate was diluted to 3 times the original blood volume with 0.0027 M EDTA-HBSS$^=$ and 20 ml was layered onto 5 ml of ice-cold Hypaque-Ficoll (9),[3] in siliconized 23- × 105-mm glass centrifuge tubes. These were centrifuged at 4°C for 20 min at 500 *g*. A layer consisting of 75–85% lymphocytes was aspirated from the interface. After two washes in 0.0027 M EDTA-HBSS$^=$, the cells were resuspended at 2×10^6 leukocytes/ml in 30% fetal calf serum (FCS) in RPMI-1640 medium (Microbiological Associates, Inc., Bethesda, Md.) containing 50 U/ml of penicillin and 50 μg/ml of streptomycin. These cells were poured into 100- × 20-mm plastic petri dishes, 10 ml per dish, and incubated at 37°C in 5% CO_2–95% air for 90 min. Nonadherent cells were obtained by pouring off the supernate and pooling this with two 37°C HBSS washes of the adherent layer. These cells were washed twice with 0.04 M EDTA-HBSS$^=$ (made with equal parts of 0.087 M EDTA stock solution and HBSS$^=$) and counted. Preparations usually contained 91–97% lymphocytes with 40–60% yield. No evidence was obtained of selective depletion of CRL or SIg-bearing lymphocytes when assays were performed before and after each purification step.

Red blood cells and granulocytes were removed from spleen and thymus cell suspensions by centrifugation through Hypaque-Ficoll by the method described above. If the resulting cell suspension contained more than 10% macrophages, adherent cells were removed on petri dishes. If viability (by trypan blue exclusion) was less than 90%, dead cells were pelleted in 24.5% bovine serum albumin (BSA) (10).

TDL represented a portion of a pool obtained from 24 h of drainage. Minor red cell and granulocyte contamination was removed by centrifugation on Hypaque-Ficoll.

Sensitized sheep erythrocytes (EA_{19S}). Sheep erythrocytes (E) (Colorado Serum Co., Denver, Colo.) were sensitized with rabbit IgM antisheep red cell antibody (Cordis Corp., Miami, Fla.) (11). Since 1.0 ml of a 1:180 dilution of this antibody per 10^9 E was found to be optimal for hemolysis by guinea pig complement, a 1:120 dilution was used routinely.

Erythrocyte-antibody-complement complexes (*EAC*). EAC1–9mo was prepared by mixing equal volumes of 2×10^8/ml EA_{19S} and normal mouse complement diluted 1:5 or 1:2.5 in gelatin veronal buffer with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (GVB). In order to produce EAC1–3mo, normal mouse complement was substituted by serum from a mouse known to be deficient in C5. Both mixtures were incubated at 37°C for 30 min, then washed 3 times in ice-cold GVB and stored at 2°C. The EAC1-9mo were used for not more than 2 days and the EAC1-3mo for up to 4 days.

Normal mouse complement was obtained from CF_1 male mice and C5-deficient mouse complement from AKR male mice. Mice were bled from the tail directly into a glass tube in an ice bath. After allowing the blood to clot for 3 h in the cold, the complement (serum) was separated by centrifugation and stored at −70°C.

$EAC14^{oxy}23hu$ cells were prepared as previously described (12), the only difference being that oxidized C2 (13, 14) was substituted for native C2. The source of C4 was either highly purified C4 or fresh human serum heated at 56°C for 30 min. $EAC14^{oxy}2$ were formed from EAC14 by addition of 90 effective molecules of highly purified oxidized C2 ($^{oxy}C2$) per cell, followed by reaction at 32°C for 13 min and 2 washes with ice-cold GVB. $EAC14^{oxy}23$ were formed by addition of 100–150 μg C3 (15) per 10^9 $EAC14^{oxy}2$, reaction at 37°C for 15 min and 3 washes with ice-cold GVB. These EAC1-3hu cells were stored at 2°C and used for up to 4 days.

Detection of CRL. Lymphocytes were suspended at 2×10^6/ml and EAC at 1×10^8/ml in 0.04 M EDTA-HBSS$^=$. Each suspension was incubated separately at 37°C for 30 min before use. 0.4 ml of lymphocytes was mixed with 0.4 ml of EAC in stoppered 12- × 75-mm siliconized glass tubes and rotated at 37°C for 15 min at 60 rpm on a tissue culture-type rotator with 9 cm radius and nearly hori-

[3] Hypaque was substituted for isopaque (triosil) in the same ratio as previously described (9).

zontal axis. Relevant controls were used and included E, EA_{19S}, and occasionally also $EAC14^{oxy}2$, substituted for the EAC. Only one batch of lymphocytes was tested at a time and nail polish-sealed slides were made immediately after taking the tubes off the rotater. Rosetted (four or more bound EAC) and unrosetted lymphocytes were counted in several fields using a Nikon phase contrast microscope (Nikon, Inc., Div. of EPOI, Garden City, N. Y.) equipped with Köhler illumination at 1000 × magnification. A total of 200–300 lymphocytes per slide was counted for each test. Whenever there was minor contamination with nonlymphocytes, an effort was made to distinguish and enumerate them. (A range of 60-90% of monocytes, granulocytes, and erythrocytes formed rosettes with EAChu.)

Immunofluorescence. Antisera directed to the γ-, α-, and μ-heavy chains and both light chain types were obtained by pooling the sera from rabbits immunized separately with human paraproteins (IgM and IgA), normal IgG and λ- and κ-Bence-Jones proteins. Conjugation of anti-immunoglobulin with fluorescein isothiocyanate and the methods used for detection of SIg were as previously described (2), with the exception that cells were incubated with fluorescein-conjugated antisera at 4°C instead of room temperature.

Combined assay for CRL and SIg. Lymphocytes were stained with fluorescein-conjugated anti-Ig antisera and then mixed with EAC to form rosettes by the method described above. Lymphocytes were simultaneously examined for both rosette formation and for fluorescent surface staining using a Leitz Ortholux microscope equipped with Ploem ultraviolet and phase contrast illumination (E. Leitz, Inc., Rockleigh, N. J.). Four types of lymphocytes were enumerated in each field: fluorescent-CRL (SIg^+CRL^+), fluorescent-non-CRL (SIg^+CRL^-), nonfluorescent-CRL (SIg^-CRL^+), and nonfluorescent-non-CRL (SIg^-CRL^-). Counts of not less than 400 lymphocytes from duplicate slides were averaged. As a control, CRL and SIg were also assayed individually.

Detection of human thymus-derived rosette-forming cells (TRFC). Lymphocytes were suspended at 3×10^6/ml and washed unsensitized sheep E at 1×10^8/ml in RPMI-1640 (Microbiological Associates, Inc.) or 0.04 M EDTA-HBSS=. 0.4 ml of each was mixed in stoppered 12- × 75-mm siliconized glass tubes and rotated for 15 min at 37°C as in the CRL assay. The suspension was pelleted at 200 *g* for 10 min and incubated as a pellet for 2 h in an ice bath. The entire pellet was resuspended by shaking the tube very gently. Sheep E rosettes (TRFC) were enumerated in the same way as described above for EAC-CRL rosettes. Three or more erythrocytes in contact with a lymphocyte was considered positive (> 90% of positive cells had five or more E on their surfaces). If viability was less than 95%, 0.2 ml of 0.1% trypan blue was added to the resuspended pellet and normal (not phase contrast) illumination was used to enumerate rosettes among viable cells only.

Combined assay for TRFC and SIg. Lymphocytes were stained with fluorescein-conjugated anti-Ig antisera and then mixed with sheep E to form rosettes by the method described above for detection of TRFC. Lymphocytes were simultaneously examined for rosette formation and fluorescent surface-staining in the same manner as for the combined assay for CRL and SIg.

RESULTS

Determination of the optimal EAC. EA_{19S} were used, since EA_{7S} were found to form rosettes with a small proportion of normal lymphocytes (1–3%). EACmo were routinely prepared with C5-deficient mouse complement (EAC1–3mo) because those prepared with normal mouse complement (EAC1–9mo) lost most of their reactivity within 2 days. A further difference between EAC1–3mo and EAC1–9mo was that only with EAC1–3mo could EDTA inhibit rosette formation with contaminating phagocytes. For this inhibition 0.04 M EDTA was required, and it was found necessary to preincubate the phagocytes in the EDTA for at least 30 min at 37°C before adding the EAC1–3mo. By this technique more than 90% rosette inhibition was achieved with suspensions of either monocytes or granulocytes. EAChu ($EAC14^{oxy}23$) prepared with purified human components was not inhibited from forming rosettes with monocytes or granulocytes in the presence of EDTA. Both EAChu and EACmo formed rosettes with human erythrocytes (immune adherence), but EAChu formed 20-fold more rosettes with human E than did EACmo.

Several batches of EAC1–3mo were prepared with different dilutions of mouse serum and tested on the same day for rosette-forming ability with several batches of normal and CLL peripheral lymphocytes (Fig. 1). A volume of 0.2–0.4 ml of mouse serum per 2×10^8 EA_{19S} was found to be optimal for detecting maximum numbers of CRL.

Similar experiments were performed with batches of EAChu containing varying amounts of C3. The results suggested (Fig. 2) that more C3 was required to detect a maximum number of leukemic CRL than was required for normal CRL. The maximum amount

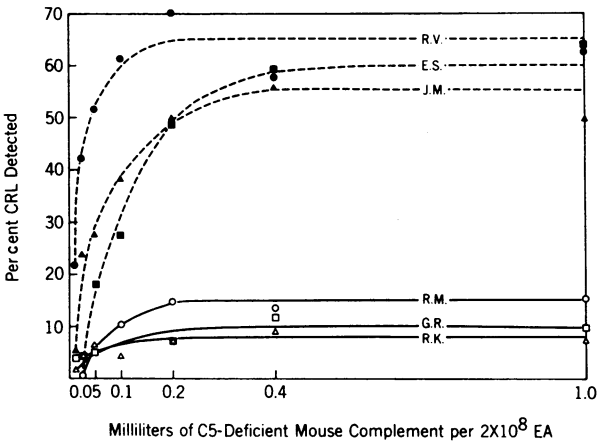

FIGURE 1 Formation of optimal EAC1-3mo for detection of CRL. Three normal individuals (solid lines) and three CLL patients (dashed lines) were tested on the same day with the same preparations of EAC1–3mo. A maximum number of CRL was detected with EAC1–3mo when they were prepared by mixing 0.2–0.4 ml of C5-deficient mouse complement (in 1.0 ml GVB) with 2×10^8 EA_{19S} in 1.0 ml of GVB.

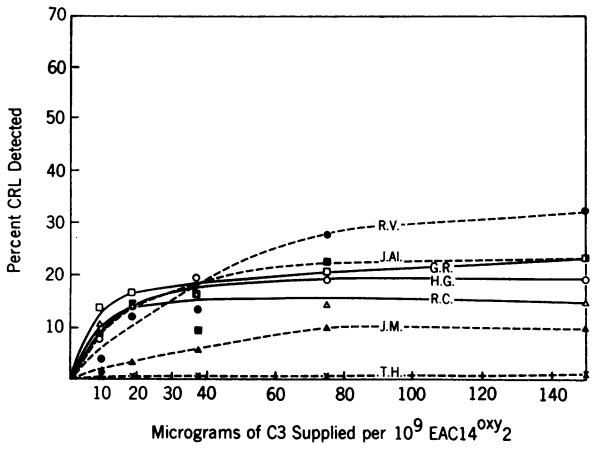

FIGURE 2 The formation of optimal EAC1–3hu for detection of CRL. Three normal individuals (solid lines) and four CLL patients (dashed lines) were tested on the same day with the same preparations of EAC1–3hu. With normal CRL, 18.8 μg of C3 per 10^9 EAC detected a maximum number of CRL but with leukemic CRL more was required.

of C3 used, 150 μg/10^9 EAC, was calculated to give approximately 50,000 molecules of bound C3 per cell. EAChu were routinely prepared with this amount of C3, since it was supraoptimal for both normal and leukemic CRL.

CRL and SIg-bearing lymphocytes in normal peripheral blood. In peripheral blood, the CRL were a subpopulation of the SIg^+ cells detected by immunofluorescence (Table I). Usually all CRL were SIg^+ and SIg^-CRL^+ cells were only rarely found in peripheral blood. In contrast, about 50% of SIg^+ cells were CRL^-. EAChu usually detected more CRL than EACmo, but on a small number of occasions (data not shown), EACmo detected the same number or slightly more CRL than did EAChu. EAC1–3mo and EAC1–9mo detected the same number of CRL. When a mixture of EAC containing half (5×10^7/ml) EACmo and half EAChu was used, an average of 14% CRL were detected. This was only slightly more CRL than the 12% detected with EAChu by itself (Table I).

CRL and SIg-bearing lymphocytes in the thymus, spleen, and thoracic duct. When TDL and spleen cells were tested, different results were found from that obtained with peripheral blood (Table II). Almost all SIg^+ cells were also CRL^+. Only 1–4% of lymphocytes were SIg^+CRL^-. In some preparations of TDL and spleen cells, a significant number of SIg^-CRL^+ cells were observed. In cells from two of the three spleens studied, 10–20% of the lymphocytes were SIg^-CRL^+. Such lymphocytes were only rarely seen in peripheral blood. These spleen cell suspensions were 80–96% lymphocytes and large leukocyte rosettes were assumed to be phagocyte rosettes and were not included in the count of CRL.

In cells from the thymus, 1–2% of the lymphocytes had a detectable C3 receptor or enough SIg to be demonstrable by immunofluorescence.

CRL and SIg-bearing lymphocytes in CLL. Several patients with CLL who had previously been studied for the presence of SIg-bearing lymphocytes (16) were restudied for CRL and SIg simultaneously (Table III). The patients were divided into two groups: one, patients who had a high percentage (24–100%) of SIg^+ lymphocytes; and the other, patients who had a low percentage (12% or less) of SIg^+ lymphocytes. A range of 10–85% CRL was detected in these patients, and no correlation was found between the presence of CRL and the presence or absence of SIg. In 8 out of 10 cases EACmo detected 2 to 20-fold more CRL than did EAChu. Increasing the amount of bound C3 on EAChu did not lead to an increase in CRL detected (Fig. 2). In three cases the percentage of CRL was similar to that observed with normal peripheral blood

TABLE I
CRL and SIg in Normal Peripheral Blood

	CRL^+		
	EACmo	EAChu	SIg^+
	%	%	%
C. A.	11	12	27
B. C.	7	11	19
R. C.	7	13	24
R. K.	8	10	23
R. M.	12	18	30
E. R.	7	10	21
G. R.	11	12	23
Average	9	12	24
Range	7–12	10–18	19–30
CRL/SIg	9/24 = 38%	12/24 = 50%	

TABLE II
CRL and SIg in the Spleen and TDL

	CRL^+		
	EACmo	EAChu	SIg^+
	%	%	%
Spleen			
1	20	22	21
2	46	49	39
3	28	39	22
TDL			
B. A.	16	ND*	12
M. B.	7	14	13
D. M.	25	25	23
P. M.	17	23	18
J. R.	27	32	26

* ND, Not Done.

(L. S., H. C., and T. H.), whereas in the remaining cases, 41–85% of the lymphocytes were CRL using EACmo, a 3–7 times higher incidence than observed with normal peripheral blood lymphocytes (Table I).

TRFC in peripheral blood and thoracic duct. In the assay of TRFC it was important to resuspend the pellet by very gentle shaking of the tube. If the pellet was resuspended by Pasteur pipette instead, 30–100% fewer TRFC were detected. When the assay was combined with trypan blue staining of dead cells, it was found that there were 2–4 times more TRFC among the viable cells. As the cells in an individual preparation died, the percent TRFC among all cells decreased, but the percent TRFC among the viable cells remained the same. It should be noted that, in contrast to the findings of Coombs, Gurner, Wilson, Holm, and Lindgren (17) and Fröland (7), a low but variable number of trypan blue staining lymphocytes were $TRFC^+$ and therefore it was not possible to accurately calculate the percent TRFC from two separate determinations of TRFC and viability. The combined assay for TRFC and viability eliminated this problem and therefore only counts of viable TRFC determined by this combined assay are given in Table IV.

Using the combined assay for TRFC and SIg, it was obvious that all (more than 99%) TRFC lacked SIg fluorescence. In peripheral blood lymphocytes 69–75% of cells were $TRFC^+$, 19–30% SIg^+, and 0–5% of cells were SIg^-TRFC^-. In TDL, as in peripheral blood lymphocytes, almost all lymphocytes could be accounted for by the simultaneous assay of the two markers (Table IV); however, since there were some B cells with only one of the two B surface markers, the total B cells had to be calculated from the percent of nonoverlap observed in the simultaneous assay of CRL and SIg:

$$\text{Total B cells} = \%\ SIg^+\ CRL^+ + \%\ SIg^+\ CRL^- + \%\ SIg^-\ CRL^+$$

For example in the case of D. $M._2$:

$$\%\ \text{B cells} = 28\% + 4\% + 3\% = 35\%$$
$$\%\ \text{T cells} = \%\ \text{viable TRFC} = 65\%$$
$$\text{Total B} + \text{T cells} = 100\%.$$

In contrast to the findings of Jondal, Holm, and Wigzell (8), no inhibition of rosette formation was observed by treating both the lymphocytes and sheep E with EDTA (0.04 M EDTA-HBSS$^=$, pH 7.2, isotonic, at 37°C for 30 min before mixing the cells and also during the assay) and therefore involvement of divalent cations in rosette formation is considered doubtful. Also, when EAC were substituted for sheep E, so that both CRL^+ and $TRFC^+$ cells could be assayed in a combined assay for SIg, 85% of peripheral blood lymphocytes were rosetted. The 15% of cells that were nonrosetted were fluorescent and therefore were accounted for by B cells that were SIg^+CRL^-. In separate assays of this peripheral blood lymphocyte preparation there were 25% of cells that were SIg^+ and 75% $TRFC^+$. The 85% of cells rosetted by EAC were thus made up of 75% TRFC (nonfluorescent rosettes) and 10% CRL (fluorescent rosettes). When the suspension of 85% rosettes was mixed by repeated pipetting with a Pasteur pipette, 10% of rosettes remained and all were fluorescent and thus SIg^+ CRL^+. This demonstrated that CRL rosettes are more difficult to disrupt than TRFC rosettes. Also, it showed that no additional CRL are detected among the SIg^+ CRL^- cells if the EAC are pelleted with the lymphocytes for closer con-

TABLE III
CRL and SIg in CLL

	SIg^+	CRL^+ EACmo	CRL^+ EAChu
	%	%	%
High surface Ig			
J. Al.	80	41	23
J. An.	100	70	18
H. C.	60	15	ND*
J. M.	70	56	10
A. O.	26	84	73
L. S.	68	10	9
W. T.	24	47	9
Low surface Ig			
T. H.	<1	10	1
E. S.	5	61	7
R. T.	12	62	3
R. V.	1	65	33

* ND, Not Done.

TABLE IV
CRL, SIg, and TRFC in Normal Peripheral Blood (NPB), TDL, and CLL

	CRL^+ EACmo	CRL^+ EAChu	SIg^+	$TRFC^+$
	%	%	%	%
NPB				
C. A.	9	11	25	75
R. K.	12	13	26	69
E. R.	8	9	25	74
TDL				
D. $M._1$	25	25	24	72
D. $M._2$	33	31	32	65
CLL				
T. H.	10	1	<1	3
J. M.	45	1	2	3
R. T.	62	3	12	3

tact. Finally, it showed that the sheep E membrane was still capable of reacting with TRFC even though it was covered with large amounts of antibody and complement. This latter finding does not invalidate the CRL assay since sheep E, EA_{19S}, and $EAC14^{0'}2$ never (< 0.1%) formed rosettes with TRFC under the conditions of the CRL assay (lymphocytes and EAC in suspension at 37°C). The formation of TRFC rosettes with EAC required pelleting of the cells and an additional incubation at 0°C for 2 h. As an additional experiment to determine whether close contact of EAC and lymphocytes would detect more CRL than the usual assay, EAC and normal peripheral blood lymphocytes were pelleted and resuspended as in the TRFC assay, and guinea pig erythrocyte EAC were used instead of sheep erythrocyte EAC, since guinea pig erythrocytes do not bind to TRFC. As in the previous experiment the assay was combined with fluorescent staining of lymphocytes for SIg. No TRFC rosettes (nonfluorescent rosettes) were observed and the number of CRL rosettes (fluorescent rosettes) was equivalent to that obtained with the normal CRL assay (EAC and lymphocytes incubated in suspension at 37°C without subsequent pelleting).

We could confirm the findings of Lay, Mendes, Bianco, and Nussenzweig (18) that it was necessary to incubate the lymphocytes at 37°C before pelleting them and incubating them at 0°C. In addition, it was found that both the lymphocytes and sheep E must be prewarmed at 37°C, but that they could be incubated at 37°C separately, cooled to 0°C, then mixed and pelleted. Contact of the sheep E and lymphocytes during the 37°C incubation was therefore not obligatory for TRFC rosette formation. It was also found that in addition to sheep E, both dog E and goat E would form rosettes with TRFC but to a lesser extent than sheep E, 50% of TRFC detected with dog E and 25% of TRFC with goat E.

TRFC in CLL. In order to further characterize the CLL patients with low percentages of SIg^+ cells (< 1–12%), studies of their capacities to form rosettes with sheep E were also done. One of these CLL patients (T. H.) also had a low percentage of CRL and therefore this patient's leukemia could have been of T cell origin. However, all three of these patients had only 3% of $TRFC^+$ cells (Table IV) as compared to 69–75% for normal individuals.

DISCUSSION

In the mouse, T and B cells can be distinguished by means of antilymphocyte sera, of either allogenic or xenogenic origin, which detect surface antigens (such as theta) on the T cell population which are not present on B cells. These differentiation antigens, however, have been shown to differ with regard to the amount of antigen present within populations of T cells, depending upon the origin of T cells; i.e., within the thymus or in peripheral organs (19). In the B cell population four other surface markers have been described: (*a*) SIg detectable by immunofluorescence and similar techniques; (*b*) a complement receptor for the third component of complement; (*c*) a receptor for the Fc fragment of IgG (20); and (*d*) surface antigens (such as MBLA) found on B cells and not T cells. As yet, no clear-cut antigenic markers have been described in the human to allow a distinction between T and B cells.

In an effort to describe the B and T lymphocyte populations in humans, SIg and the complement receptor have been used to identify B cells and the recently described sheep E rosette-forming capacity used to identify T cells (6–8). Lymphocytes from normal individuals and individuals with CLL were studied. In human peripheral blood as much as 50% of lymphocytes that are SIg^+ are CRL^-. In contrast in the spleen there appear to be some cells that are CRL^+ and SIg^- (10–20%). In agreement with this we have found in mouse peripheral blood 14–20% of lymphocytes are SIg^+, whereas only 7–11% are CRL^+. (Ross, G. D., and E. M. Rabellino. 1972. Unpublished observation). There may also be a small population (5–10%) of B cells that are SIg– in the mouse. (Vassalli, P. 1972. Personal communication). Thus, at the present time in the human, as well as in the mouse, it would appear that neither CRL nor SIg is an altogether reliable marker for B cells. The situation appears to be quite analogous to that described for differentiation antigens, in that at different stages of differentiation and in different lymphocyte populations, more or less of these antigens may be present on the surface of cells and, depending upon the detection system, may or may not be detectable.

Pincus, Bianco, and Nussenzwig (21) and Shevach, Herberman, Frank, and Green (22) have recently reported similar numbers of CRL in human peripheral blood as we observed. Jondal et al. (8), on the other hand, reported nearly twice as many CRL in peripheral blood, with nearly equal numbers of CRL and SIg cells. In our combined simultaneous study of both markers on the same cells, we always found that 50% or more of the fluorescent (SIg^+) lymphocytes from peripheral blood were CRL^-. Jondal et al. (8) assayed CRL and SIg separately, and therefore could not have seen the significant numbers of SIg^+ CRL^- lymphocytes that we observed. All of our data given in Table I were determined by combined assay for CRL and SIg. We also confirmed the finding of Bianco and Nussenzweig (23) that anti-immunoglobulin treatment of lymphocytes did not affect the detection of CRL. In order to try to find the reason for the discrepancy between the data of Jondal

et al. (8) and our own, we tried to duplicate their CRL assay. We used guinea pig erythrocyte EAC, instead of the human erythrocyte EAC that they used and obtained the same number of CRL^+ cells as we did by our standard assay. The use of human red blood cells as the indicator cells in the CRL assay would appear to be hazardous since human erythrocytes themselves have C3 receptors (immune adherence receptors) (24), and the human EAChu cells would be expected to form rosettes among themselves or with any contaminating human erythrocytes in the lymphocyte preparation. Guinea pig erythrocytes do not have immune adherence receptors and do not bind to TRFC. It should, however, be noted that sheep erythrocyte EAC do not bind to TRFC under the conditions of the CRL assay (37°), but do bind under the conditions of the TRFC assay (0°C). Pincus et al. (21) have reported similar findings with sheep erythrocyte EAC binding to TRFC. If the CRL assay is always conducted at 37°C, sheep erythrocyte EAC only detect CRL and all CRL are detected with the cells in suspension with no need for pelleting of the cells for better contact. In TDL, for example, almost all $TRFC^-$ cells are CRL, and therefore, the assay of CRL without pelleting detects the maximum number of CRL. We therefore offer two possible explanations for the discrepancy between the data of Jondal et al. (8) and our own: (*a*) their use of separate assays for CRL and SIg did not allow them to detect the large number of SIg^+ CRL^- that we always detect by our routine simultaneous assay for CRL and SIg; (*b*) their use of human red blood cells for indicator cells may have caused many rosettes among the indicator cells or contaminating red cells, which were occasionally mistaken for CRL rosettes. In our own studies, we have found that 1000 × magnification and phase contrast illumination are necessary to distinguish human red cell rosettes from CRL rosettes.

The discordancy between CRL and SIg-bearing lymphocytes in normal lymphocytes is further accentuated by the studies on CLL cells. In previous studies it was shown that the great majority of individuals with CLL contained lymphocytes with monoclonal IgM on their surface, while a few cases had only light chains detectable, and others, 10–20% of the total cases, had no detectable SIg (16, 25). Since these latter cases were potentially T cell leukemias, due to their lack of detectable SIg, special emphasis has been placed on the study of these individuals. Two findings have emerged from this study. First, using EACmo, it was clear that the lack or presence of SIg was not correlated with the lack or presence of CRL. Instances were found where the great majority of cells were positive for SIg and a relatively low percentage were CRL and conversely where the great majority of cells were negative for SIg and yet were highly positive for CRL. These studies indicate that those leukemias which lack easily detectable SIg are still probably of B cell origin since three out of four examined had a large percentage of CRL. This suggestion is further strengthened by studies that have been performed on quantitative analysis of the amount of immunoglobulin associated with the surface and cytoplasm of these cells. Although by immunofluorescence these cells were negative, when a sensitive radioimmunoassay was performed, all of these patients had detectable amounts of IgM on the surface (26). Furthermore, in freeze-thaw lysates containing the total surface and cytoplasmic immunoglobulin, it was demonstrated that similar amounts of immunoglobulin were associated with the cells that lacked detectable SIg by immunofluorescence, as compared to those which contained it. Also, these amounts of immunoglobulin are only slightly less than those found in normal B cells and are much larger than any amount of immunoglobulin which has been previously detected in T cell populations (*26, 27*).

The second finding to emerge from the study of CRL in CLL was that the leukemic cells reacted weakly with human C3 compared to mouse C3. This was in sharp contrast to normal human peripheral blood lymphocytes with which fairly similar data were obtained with the two sources of complement, human complement detecting slightly more CRL than mouse complement. With CLL, mouse complement detected equal numbers (2 out of 10 patients) or considerably larger numbers of CRL (8 out of 10 patients) than did human complement. The reason for this discrepancy between leukemic and normal lymphocytes is at present totally unknown, but it is suggestive that an altered complement receptor may be present on the leukemic cells. Furthermore, our data demonstrate that patients with low SIg do not contain appreciable amounts of TRFC. These data are similar to those obtained by others who studied TRFC in CLL patients with a high percentage of SIg^+ cells (*7, 8*).

Previously, four other studies of CRL in CLL had been reported (*21, 22, 28, 29*). Michlmayr and Huber (*28*) and Nishioka (*29*) reported that CLL patients had fewer CRL than normal individuals. Pincus et al. (*21*) and Shevach et al. (*22*) reported that CLL patients had far more CRL than normal. These apparently conflicting results can now be explained by the use of different complement sources. Michlmayr used EAChu, Nishioka used EACgp, while Pincus and Shevach used EACmo. It is obvious from our results that the differences between Michlmayr's, Pincus's and Shevach's results are explainable on the basis of the different capacity of EAChu and EACmo to react with CLL lymphocytes. Since Nishioka used enough guinea pig C3

to detect normal CRL, it is likely that guinea pig C3, like human C3, reacts poorly with leukemic cells. Since human C3 did not react at all with some CLL cells, it was of interest whether EACmo reacted with CLL cells through mouse C3 and not by some other means. It was found that Fab fragments of antimouse C3 completely inhibited EACmo rosettes with CLL cells, assuring that mouse C3 was indeed the means of attachment of EACmo to CLL cells (Ross, G. D. 1972. Unpublished observation).

Recently several investigators have reported that human T lymphocytes form rosettes with sheep erythrocytes. In the early studies only a relatively small population of peripheral blood lymphocytes formed rosettes (6, 7, 18, 30); however, with some modifications in the technique, the studies of Jondal et al. (8) as well as our own clearly indicate that the reaction between sheep erythrocytes and human lymphocytes is a quantitative marker for T cells and that in combination with markers for B cells such as SIg and CRL, all the lymphocytes in normal peripheral blood and thoracic duct can be accounted for.

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