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Distribution of Complement Receptors on Human Normal and Malignant Mononuclear Cells

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Mononuclear cells from normal human subjects and patients with chronic lymphocytic leukemia (CLL), chronic lymphosarcoma cell leukemia (LCL), and hairy cell leukemia (HCL) were labeled with fluoresceinated, purified human C3b (Fl-C3b) and analyzed using the fluorescence-activated cell sorter (FACS). Fl-C3b labeled 17.6% ± 6.0% of peripheral blood mononuclear cells (PBM) from 20 normal subjects, which, when separated by the FACS, consisted of B lymphocytes and approximately 5% monocytes. Analyses in which either monocytes or B lymphocytes were excluded from consideration demonstrated that both these cell types were labeled by the Fl-C3b with a heterogeneous distribution of fluorescence intensity, indicating either heterogeneity of CR density or variable avidity of individual CR for the Fl-C3b. FACS profiles of PBM (<5% monocytes) from 14 of 15 patients with CLL showed a homogeneous distribution of very low fluorescence intensity, with >60% of the cells being slightly more fluorescent than unlabeled controls. This low, homogeneous distribution of fluorescence is strikingly similar to profiles of CLL cells labeled with anti-Ig reagents and suggests homogeneity of low CR density and/or avidity. Similarly, CR^+ mononuclear cells from five patients with HCL and three patients with LCL displayed more homogeneous Fl-C3b labeling than normal CR^+ PBM. Homogeneity of Fl-C3b binding to CLL, LCL, and HCL cells further supports the concept for a clonal origin for these disorders.

RECEPTORS THAT BIND certain components of complement have been identified on many cell types, including lymphocytes, monocytes, and malignant cells from patients with lymphoproliferative diseases. Cells bearing complement receptors (CR) can be distinguished from CR^- cells by their ability to form rosettes with complement-coated particles, such as IgM-sensitized sheep erythrocytes (SRBC) or gram-negative bacteria.[1,2] Human lymphocytes may bear CR of two distinct specificities, designated CR_1 and CR_2.[3] CR_1, the immune adherence receptor, binds native C3, C4, and C5 proteins weakly and their activated b forms more strongly.[4] CR_2 binds C3d, and may bind C3b after conformational change by β_{1H}-globulin or after conversion to C3bi by C3b inactivator. CR_2 is also reported to bind fluid phase C3 and C3b, presumably because of a degree of unfolding of the protein chains.[5] A third CR type (CR_3) has been identified on monocytes and granulocytes, and like CR_2, binds C3b after interaction with C3b inactivator and β_{1H}-globulin, though at a different site on the C3 molecule.[4]

The rosette techniques used to identify and quantitate CR^+ cells unfortunately give no information regarding the cell-to-cell distribution of CR density or avidity. Using fluid phase mouse C3b conjugated to fluorescein isothiocyanate (FITC), other investigators have shown that murine and human CR-bearing lymphocytes can be identified with conventional fluorescence microscopy.[6] The present study reports the analysis of FITC-conjugated human C3b-labeled mononuclear cells by a fluorescence-activated cell sorter (FACS-II, Becton-Dickinson, Linden, N.J.). This technique allows not only for assessment of the frequency of CR-bearing cells, but also provides data regarding the distribution of cell-to-cell C3b binding.

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Supported in part by Naval Medical Research and Development Command, Research Task ZF51.524.013.1025 and National Naval Medical Center (Clinical Investigation 7-06-1073).

The opinions and assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

Submitted December 7, 1979; accepted June 30, 1980.

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0006-4971/80/5605-0007$01.00/0

MATERIALS AND METHODS

Human Subjects

Heparinized venous blood was obtained from healthy adult volunteers and from patients with chronic lymphocytic leukemia (CLL), chronic lymphosarcoma cell leukemia (LCL), hairy cell leukemia (HCL), and nodular poorly differentiated lymphocytic lymphoma (NPDL). Diagnoses were confirmed by histopathology of peripheral blood, bone marrow, and in some cases, lymph node and spleen. Patients with HCL all had cells that demonstrated tartrate-resistant acid phosphatase activity. Spleen cells from four normals and from three patients with HCL were also studied, as were thymocytes from an infant undergoing cardiac surgery.

Preparation of Reagents

Human C3 was prepared from pooled, citrated plasma,[7] the native protein subjected to partial proteolysis, and the resultant C3a and C3b fragments separated by molecular sieve chromatography under acid conditions.[8] The C3b was free of contamination as ascertained by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis versus anti-whole human serum. Immunization of rabbits with this C3b preparation, via footpad injection in Freund's complete adjuvant, resulted in production of monospecific antibody to the C3b protein (a single precipitin arc was formed in immuno-

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electrophoresis against whole human serum). The C3b was then conjugated with FITC (Eastman, Rochester, N.Y.) to produce a fluorescein:protein ratio of approximately 1.2.[9]

Flourescein-conjugated $F(ab')_2$ fragments of affinity chromatography-purified anti-human μ, γ, and Fab were prepared as previously described.[10]

Cell Preparation and Labeling

Peripheral blood mononuclear cells (PBM) and spleen cells were obtained by Ficoll-Hypaque density gradient centrifugation.[10] The frequency of monocytes, determined by counting 500 cells after staining for nonspecific esterase activity,[11] was 5%–25% of 20 preparations of normal PBM, 5% of normal spleen, and less than 5% of PBM from patients with CLL, LCL, and HCL. Incubation of normal PBM with 5% carbonyl iron (GAF, Lyndon, N.J.) prior to gradient centriguation reduced the frequency of monocytes to less than 1%, but also substantially reduced the B lymphocyte population.[10]

After washing twice in RPMI-1640 with 10% fetal calf serum (FCS) (GIBCO, Grand Island, N.Y.) and resuspending in RPMI-1640 with 10% FCS and 1 mg/ml sodium azide (NaN_3) at a concentration of 5×10^7 cells/ml, the mononuclear cells were incubated at 4°C for 30 min with concentrations of the fluoresceinated reagents that had proved optimal in previous experiments (1 mg/ml for the Fl-C3b). Preliminary studies indicated that FCS was important for optimal labeling and cell viability. This material, though heat-inactivated, was found to contain approximately 10% of the β_{1H}-globulin and C3b inactivator activity of normal plasma at 37°C, using the method of Gaither et al.[12] However, the conversion of C3b to C3bi and C3d is known to be temperature-dependent, occurring efficiently only at near-physiologic ranges.[12] Cells in this study were labeled and washed at 4°C, and essentially no conversion to C3bi or C3d was demonstrated at this temperature. Furthermore, preliminary analyses demonstrated equivalent Fl-C3b labeling of cells in the presence of either FCS or crystallized bovine serum albumin (BSA), which contains no β_{1H} or C3b inactivator. After labeling, the cells were washed twice in cold RPMI with 10% FCS and 0.1% NaN_3, and analyzed with the FACS.

Fluorescence-Activated Cell Sorter Analysis

Cells were analyzed in the cold within 2 hr of labeling using optimal FACS sensitivity gain settings that were determined for each reagent in preliminary experiments. FACS analyses of 20,000 cells were displayed as fluorescence profile histograms, with linearly increasing fluorescence on the X-axis and the relative number of cells within each fluorescence channel on the Y-axis. Analysis of light scatter, which is a property of a number of variables including cell size and refractive index, was used to delineate viable mononuclear cells for fluorescence analysis. Previous studies[10] demonstrated a characteristic bimodal distribution of light scatter when viable PBM were examined, with peaks of small and large cells, the latter comprising about 20% of normal PBM and being mostly esterase-positive. When the larger cells were excluded from FACS consideration, only about 5% of the remaining cells were monocytes. Therefore, for these studies, in order to assess the Fl-C3b binding of lymphocytes, large cells were routinely excluded from fluorescence analysis.

Rosette Studies

EAC43b were prepared using purified 19S antibody and human complement components as previously described,[12] and rosette formation was determined by standard methods[13] in the absence of serum.

Specificity of the Fl-C3b

Human erythrocytes (E), which bear weak Cr_1 but no CR_2 or CR_3, were used to assess the CR_1 binding of F1-C3b. FACS analysis of Fl-C3b-labeled E revealed that more than 80% of these cells had higher fluorescence intensity than unlabeled E, thereby demonstrating that the Fl-C3b could bind to CR_1.

RESULTS

CR Profiles in Normal Subjects

Mononuclear cells derived from the peripheral blood of 20, the spleens of 4, and the thymus of 1 normal individual(s) were studied. By FACS analysis, Fl-C3b labeled 17.6% ± 6.0% of PBM, 38.3% ± 8.6% of spleen cells, and 3.3% of thymocytes. A representative fluorescence profile of Fl-C3b labeled PBM is shown in Fig. 1A. This profile consists of a homogeneous peak of labeled cells (solid line) with the same low fluorescence intensity as unlabeled cells (dotted line) and therefore CR negative, and a second population with much brighter and more heterogeneous fluorescence intensity. The latter population was considered CR positive. Each of the 20 normal PBM profiles demonstrated similarly heterogeneous CR^+ cell fluorescence, as did the four spleen cell profiles, represented by Fig. 1C.

Since PBM and spleen cell preparations contained both lymphocytes and monocytes, it was necessary to determine if the fluorescence heterogeneity of CR^+ cells was the result of heterogeneous Fl-C3b labeling of one or both cell types. Two approaches were utilized to study this issue. In the first, PBM were obtained from four patients with nodular lymphoma who had

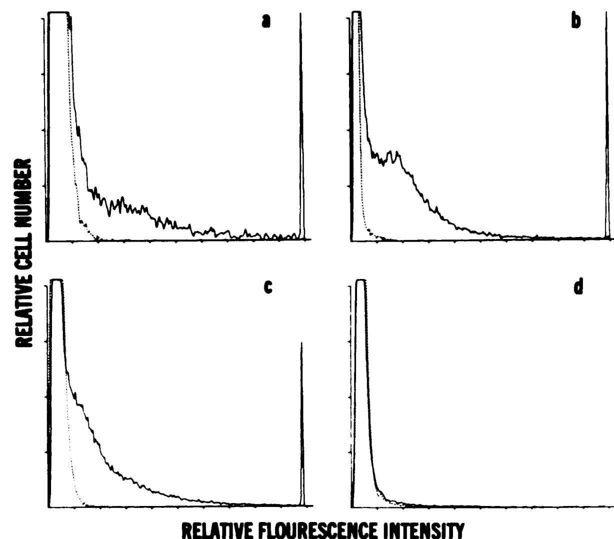

Fig. 1. Representative fluorescence profiles of Fl-C3b-labeled cells (solid lines) and unlabeled cells (dotted lines) obtained from: (A) normal PBM, (B) PBM from a B-lymphocyte-depleted patient with nodular lymphoma, (C) normal spleen, and (D) normal thymus.

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been treated with low-dose total body irradiation. This procedure results in the ablation of circulating B lymphocytes, and when CR^+ PBM from these patients were separated by sorting, more than 95% were esterase-positive. Therefore, fluorescence profiles primarily reflected the distribution of Fl-C3b labeling on monocytes. These profiles, an example of which is Fig. 1B, were similar to those of normal PBM and spleen, showing a heterogeneous distribution of CR^+ fluorescence intensity. The second approach involved the in vitro depletion of monocytes by incubation of PBM with carbonyl iron. This procedure reduced the frequency of monocytes to less than 1% of the total PBM, and although some B lymphocytes were also depleted, the remaining CR^+ cells, after Fl-C3b labeling, displayed heterogeneous fluorescence intensity (data not shown). Therefore, both lymphocytes and monocytes were shown to have heterogeneity of Fl-C3b binding.

To determine the frequency of peripheral blood lymphocytes that bound the Fl-C3b, PBM from three normal subjects were separated by sorting into CR^- and CR^+ fractions. Each fraction was then stained for nonspecific esterase activity and the frequency of monocytes determined. The frequency of CR^+ lymphocytes was then calculated according to the following formula:

$$\% CR^+ \text{ lymphocytes} = \frac{\% CR^+ PBM - \% CR^+ \text{ monocytes} \times 100}{100 - \% \text{ total monocytes}}$$

Results of these three experiments are listed in Table 1. A mean frequency of 13.4% was calculated for CR^+ peripheral blood lymphocytes, while a mean of 85% (range 76%–100%) of the peripheral blood monocytes were CR^+ by this technique. These data were obtained by analyzing only cells whose light scatter was in the viable lymphocyte range. When only larger cells (monocyte light scatter range) were analyzed and sorted into CR^+ and CR^- fractions, more than 90% of the CR^+ cells were esterase-positive monocytes and less than 10% were lymphocytes.

Table 1. Frequencies of Normal PBM Lymphocytes and Monocytes in Analyzed Samples and Sorted CR Fractions

	Frequency of Cells Analyzed (%)			
Experiment	All CR^+	CR^+ Monocytes	All Monocytes	Calculated Frequency of CR^+ Lymphocytes*
1	17.1	3.6	3.6	14.0
2	21.9	6.9	9.1	16.5
3	13.0	3.7	4.0	9.7
Mean	17.3	4.7	5.6	13.4

*Percent CR^+ lymphocytes = % CR PBM − % CR^+ monocytes × 100/100 − % total monocytes.

Additional sorting experiments were performed to determine if CR^+ cells were coincident with sIg^+ cells in populations of PBM. Cells derived from normals were labeled with fluorescein-conjugated anti-human μ, γ, or Fab, and analyzed with the FACS. The anti-μ reagent has previously been shown to label more than 90% of sIg-bearing lymphocytes, but not monocytes.[10] The anti-γ labels monocytes, presumably by binding to cytophilic antibody, but only about 1% of peripheral lymphocytes.[10] The anti-Fab reagent labels all classes of sIg and therefore identifies both monocytes and sIg-bearing lymphocytes.[10] Cells that did not bind the anti-μ, -γ, or -Fab were separated by sorting, washed, labeled with Fl-C3b, and reanalyzed with the FACS. CR fluorescence profiles of μ^- cells revealed a population of CR^+ cells whose FACS light scatter characteristics corresponded to those of monocytes, while γ^- CR^+ cells had light scatter characteristics of lymphocytes. Fluorescence profiles of Fab^- PBM labeled with Fl-C3b demonstrated fewer than 1% CR^+ cells, suggesting that the Fl-C3b and anti-Fab reagents for the most part labeled the same population of PBM.

PBM from 15 patients with CLL, 3 patients with LCL, and 2 patients with HCL were studied and contained fewer than 5% monocytes and variable frequencies of morphologically abnormal lymphocytes. In 3 additional cases of HCL that demonstrated severe leukopenias, spleen cells were analyzed.

Representative fluorescence profiles of Fl-C3b-labeled CLL cells from 2 patients are shown in Fig. 2A and B. Fourteen of 15 CLL patients had profiles similar to those shown, demonstrating a single narrow peak of low-intensity fluorescence, the mean of which

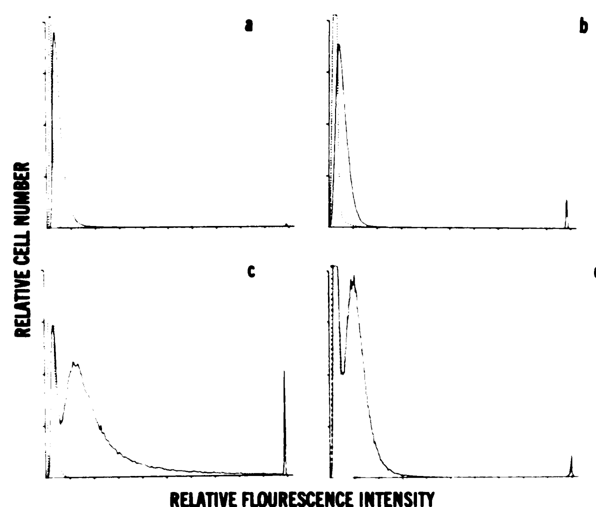

Fig. 2. Representative fluorescence profiles of Fl-C3b-labeled cells (solid lines) and unlabeled cells (dotted lines) obtained from peripheral blood of patients with CLL (A and B), LCL (C), and HCL (D).

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was several-fold lower than that of normal CR^+ PBM. Although separate peaks of CR^- and CR^+ cells could not be discerned, over 60% of the cells from each of the 14 patients were more fluorescent than unlabeled cells (Table 2). CLL patient no. 15 had only 2% Fl-C3b labeling by FACS analysis, and more than 90% of his PBM bore receptors for sheep erythrocytes, supporting the diagnosis of T-cell CLL. EAC43b rosette results from 10 of the CLL patients are listed in Table 2. Cells from patients 1–9, >60% of which bound Fl-C3b, formed rosettes in frequencies ranging from 59% to 98%. Patient no. 15 had <1% EAC43b rosetting cells.

LCL, a disorder that is clinically similar to CLL, is characterized by extremely high density sIgM, as opposed to the low homogeneous density of sIg on CLL cells.[10,14] Fl-C3b labeling density on LCL cells from three patients varied from low to high, but in all three instances was more homogeneous than normal PBM. A representative profile of Fl-C3b-labeled LCL cells as compared with unlabeled cells is shown in Fig. 2C. The frequencies of CR^+ cells by both Fl-C3b FACS analysis and by EAC43b rosettes are listed in Table 2 and were similar to those of $sIgM^+$ PBM in these patients.

CR fluorescence profiles of peripheral blood or spleen cells from 5 patients with HCL also demonstrated high frequencies of Fl-C3b labeling, as shown in Fig. 2D, with CR^+ cells ranging from 48% to 95% (Table 2). Definite peaks of CR^- and CR^+ cells were discernible in 4 of the 5 HCL profiles, and the distribution of CR on receptor-bearing cells was more homogeneous than that of normal PBM or spleen. As in the LCL cases, mean fluorescence intensity of HCL CR^+ cells varied widely among the patients studied. EAC43b rosette analysis of spleen cells from one HCL patient demonstrated a similar frequency of rosettes to that of Fl-C3b labeling.

Table 2. Frequency of F1-C3b-Labeled* and EAC43b-Rosetting Cells From Pateints With CLL, LCL, and HCL

Patient	Source	Diagnosis	Frequency (%)	
			F1-C3b*	EAC43b*
1	PB†	CLL	>60	59
2	PB	CLL	>60	91
3	PB	CLL	>60	98
4	PB	CLL	>60	84
5	PB	CLL	>60	86
6	PB	CLL	>60	82
7	PB	CLL	>60	85
8	PB	CLL	>60	60
9	PB	CLL	>60	69
10	PB	CLL	>60	ND‡
11	PB	CLL	>60	ND
12	PB	CLL	>60	ND
13	PB	CLL	>60	ND
14	PB	CLL	>60	ND
15	PB	CLL	2	<1
16	PB	LCL	63	81
17	PB	LCL	64	88
18	PB	LCL	>60	62
19	Spleen	HCL	84	76
20	Spleen	HCL	95	ND
21	Spleen	HCL	68	ND
22	PB	HCL	52	ND
23	PB	HCL	48	ND

*Analyzed by the FACS.
†Peripheral blood.
‡Not done.

DISCUSSION

This report presents a new technique for the analysis of cell-to-cell binding of fluid phase C3b. Differences in CR avidity and/or density among cell populations can be directly assessed and the frequency of CR^+ cells quantitated. However, it must be emphasized that this technique is primarily intended as a method to analyze relative density of C3b labeling. In fact, it is suboptimal for the precise quantitation of the frequency of CR^+ cells in CLL, since no clear distinction could be drawn between CR^- and CR+ populations by the FACS. These data do indicate, however, that in many cases normal PBM can be distinguished from malignant lymphocytes on the basis of C3b labeling density as well as degree of CR heterogeneity.

Since lymphocytes bear at least two types of CR, it is important to determine which of these bound the Fl-C3b. Although only CR_1 binding might be expected, the presence of FCS, which is known to contain β_{1H}-globulin and C3b inactivator, introduced the possibility that some or all of the C3b was converted to C3bi or C3d and thereby preferentially bound to CR_2. In order to specifically address this question, assays for β_{1H}-globulin and C3b inactivator[12] were performed, duplicating the Fl-C3b labeling conditions. Though the FCS contained approximately 10% of the β_{1H} and C3b inactivator activity of normal plasma at 37°C, virtually no activity could be demonstrated at 4°C, which was the labeling and washing temperature used in this study. Furthermore, since equivalent PBM labeling was obtained using crystallized BSA, and since human erythrocytes bind the Fl-C3b in the presence of FCS, it seems likely that CR_1 binding was predominant. However, since it is reported that fluid phase purified C3 and C3b may interact with C3d as well as C3b receptors,[5] a small amount of CR_2 labeling cannot be excluded.

The binding of Fl-C3b to both normal peripheral blood lymphocytes and monocytes was quite heterogeneous, suggesting either marked differences in cell-to-cell CR density or variable receptor avidity.

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However, it cannot be ascertained from these experiments whether individual CR bind Fl-C3b with constant avidity or whether a single cell has a range of receptor avidities.

The frequency of F1-C3b-labeled normal PBM in this study was somewhat lower than that of CR_1^+ cells found by other investigators using rosette techniques,[15] and is indeed lower than the frequency of EAC43b-rosetting PBM normally found in this laboratory (20%–30%). However, normal PBM usually contain 15%–25% monocytes, most of which form EAC43b rosettes, and in this study the frequency of monocytes analyzed by the FACS was only about 5%. Furthermore, the calculated mean frequency of Fl-C3b-labeled peripheral blood lymphocytes in the three sorting experiments was 13.4%, only slightly less than the 17.0% average found by Ross et al.[15] This difference may simply be due to the small number of experiments reported here. Perhaps, however, it may be related to the fact that in rosette assays not all monocytes can be identified and excluded from analysis, since 5%–10% of these cells may fail to ingest latex particles and be incorrectly considered to be CR_1^+ lymphocytes. Clearly, FACS analysis, by allowing one to look at the heterogeneity of binding sites, generates a different type of information than does red cell binding studies. Because the red cell rosette assays are simple to perform, both have a place in this type of analysis.

The Fl-C3b in these experiments labeled only monocytes and sIg^+ lymphocytes. Essentially no labeling was found when sorted sIg^- cells were incubated with Fl-C3b. These data are in conflict with those of Ross et al.,[15] who found a small number of $sIg^-CR_1^+$ cells in the peripheral blood of normal individuals. These $sIg^-CR_1^+$ cells were heterogeneous with respect to the presence of Fc receptors (FcR), Ia antigens, and receptors for sheep erythrocytes. There are several possible explanations for these conflicting data. A small population of CR_1^+ B lymphocytes may exist that bears scanty enough sIg to go undetected by flourescence microscopy, but are seen as sIg^+ by the more sensitive FACS. Alternatively, some FcR^+ sIg^- CR_1^+ lymphocytes may have passively acquired cytophilic IgG and been labeled by the anti-Fab used for FACS analyses. These cells would then have been sorted out of the Fab fraction and would not have been available for Fl-C3b labeling. Finally, it is possible that despite very rigorous efforts to exclude monocytes from consideration, the cells analyzed by Ross and coworkers may have included a few contaminating CR_1^+ monocytes. In any case, the experiments presented in this report, though not confirming a small CR_1^+ sIg^- lymphocyte population, certainly cannot exclude its existence.

Fl-C3b labeling of CLL cells was distinctively scanty and homogeneous, suggesting either uniformly low CR density or low receptor avidity. This finding supports the data of Ross and coworkers,[16] who found that optimal EAC1-3b rosettes on CLL cells required a fourfold increase in the amount of C3 used. However, Ross et al.,[3,16] using optimal amounts of C3, still found substantially lower frequencies of EAC43b-rosetting CLL cells than were reported here by either rosette or Fl-C3b techniques. The reasons for this discrepancy are unclear, but may be related to differences in rosette assay sensitivity, or perhaps may simply reflect sampling error. If, as is generally believed, CLL is a clonal disease, one would not expect a mixed population of CR_1^+ and CR_1 cells. Rather, it is more probable that some rosette techniques are relatively insensitive to the sparse or weak CR_1 on CLL lymphocytes. CR_2, which are usually detected in higher frequencies than CR_1 in parallel rosette assays on CLL cells,[3,16] may simply be more numerous or more avid than CR_1. The homogeneous, low fluorescence intensity Fl-C3b labeling on CLL cells was strikingly similar to their labeling by anti-Ig antisera.[10]

The Fl-C3b labeling of LCL cells was quite variable in terms of mean fluorescence intensity among the three patients studied, ranging from much lower to slightly higher than that of normal PBM. Despite their variability of Fl-C3b fluorescence intensity, cells from all three LCL patients demonstrated much more homogeneous labeling than normal PBM, suggesting uniformity of cell-to-cell CR avidity or density. HCL CR^+ cells similarly demonstrated homogeneous but variable mean intensity of Fl-C3b labeling. This homogeneity of Fl-C3b binding on cells from patients with CLL, LCL, and HCL is similar to their homogeneity of sIg,[10,17] and further supports the concept of a clonal origin for these disorders.

These data indicate that analysis of Fl-C3b-labeled mononuclear cells with the FACS is a simple, useful technique for the assessment of complement receptor binding, particularly with regard to comparison of C3b density between normal and malignant mononuclear cell populations. While this technique will not replace rosette assays for simple quantitation of CR-bearing cells, it does provide additional data regarding CR density and/or avidity, which rosettes can only indirectly estimate.

ACKNOWLEDGMENT

The authors wish to thank Drs. Richard Wistar, Jr. and Melvin Berger for providing reagents; Thelma Gaither for performing assays of β_{1H} and C3b inactivator activity and providing EAC43b; Richard E. Budd and Robert Habbersett for technical assistance; and Caylor Bowen for editorial support.

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