The B Cell IgM Fc Receptor: Further Evidence for the B Cell Origin of "Null" Chronic Lymphocytic Leukemia

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Fifty-three cases of chronic lymphocytic leukemia (CLL) were studied for the presence of the B cell IgM Fc receptor (Fc μR) using an aggregated IgM reagent. Restricted surface immunoglobulin, using conventional immunofluorescent techniques and FACS analysis, was detected in 43 cases (81%). The cells in the remaining ten cases (19%) expressed negligible surface immunoglobulin (sIg^-) and did not form E rosettes (E^-), but this "null" subset clearly expressed the B cell Fc μR. The coincident membrane expression of the B1 antigen and the Ia-like antigen, as well as serial studies showing surface membrane light chain acquisition (in one patient), provided additional evidence for the B cell origin of this sIg^-E^- subset. This subgroup of CLL appears to correspond phenotypically to a normal counterpart at a stage of B cell differentiation between the pre-B cell and the $sIgM^+$ early B cell. The B cell Fc μR appears to be a consistent and potentially useful marker for sIg^-E^- ("null") CLL.

WITH THE DEVELOPMENT of new reagents to detect cell surface membrane structures, the cellular origin of lymphoid neoplasms has been remarkably clarified. The chronic lymphocytic leukemia (CLL) cell yielded early to definition, as the majority of cases expressed clearly definable, albeit low density, monoclonal surface immunoglobulin.[1] The typical CLL cell expresses surface IgM (sIgM), often associated with sIgD, as well as receptor for C'3 and the Fc fragment of IgG (Fc γR). Monoclonal hybridoma antibodies directed against surface antigens such as HLA-DR (Ia) will also react with most cases and lend further support to the B cell origin of sIg^+ cases. There have been several inconsistencies observed, however. Early work by Foon and associates[2] reported the simultaneous presence of T-associated differentiation antigens on sIg^+ cells, and more recently, several hybridoma antibodies directed against T-associated differentiation antigens clearly cross-react with a substantial subset of sIg^+ CLL.[3] The meaning of these observations awaits further clarification.

In addition to these uncertainties, there are a number of sIg^- cases that also fail to display T-associated antigens and have clearly been present in several series, including our own. When the observation was first made, it was reasoned that since sIg density in the typical case is low, these "null" cases express densities of sIg below the limits of detectability by conventional fluorescence microscopy. The development of highly sensitive laser-based flow cytometers for detecting surface immunoglobulin has failed to substantiate this view,[4] suggesting that sIg^-E^- cases do indeed exist.

In this study, we have examined a substantial number of sIg^-E^- CLL cases for the presence of the new B lineage-associated Fc receptor for IgM (Fc μR) as well as for the presence of T- and B-associated differentiation antigens detected by hybridoma antibodies. Our data support the view that sIg^-E^- ("null") CLL is of B cell lineage in the vast majority of instances.

MATERIALS AND METHODS

Patient Selection

Since 1977, cell isolates from 53 patients with CLL seen at Tufts-New England Medical Center and its associated hospitals have been referred for surface receptor phenotype determinations. The diagnosis of CLL in each case was based on standard clinical and morphological criteria.[5] Certain patients were studied sequentially over a number of years and had serial surface phenotype determinations.

Preparation of Mononuclear Cell Suspensions

Mononuclear cells were isolated from heparinized peripheral blood (PB), bone marrow (BM), or freshly obtained biopsy samples of lymph nodes (LN) by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients. Blood or bone marrow was layered directly onto the gradient after dilution with saline, while tissue from lymph nodes was minced until a single-cell suspension was obtained before layering onto the gradient. Monocyte contamination of cells isolated from the gradient was assessed by morphological appearance and latex particle phagocytosis. No cell population had monocytes in excess of 5%. Viability of the cells in suspension was assessed by trypan blue dye exclusion, and in all cases, viability exceeded 90%. All studies were performed on fresh cells, with the exception of the studies utilizing the monoclonal antibody B1, which were performed on cells that had been frozen at −70 °C in a Revco freezer in Hanks' balanced salt solution, 40% fetal calf serum, and 10% dimethylsulfoxide.

Prior to each assay, cell isolates were incubated at 37 °C for one-half hour to rid cells of cytophilic antibody.

Preparation of Fluorescent-Labeled IgM Aggregates

Rabbit IgM was purified from pooled rabbit serum by sequential saturated ammonium sulfate precipitation, gel chromatography, and

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affinity chromatography, as described previously.[6] Purified rabbit IgM (10 mg/mL) in phosphate-buffered saline (PBS, pH 7.0) was then conjugated to fluorescein isothiocyanate (FITC), and excess FITC was removed by Sephadex G-25 chromatography (Pharmacia Fine Chemicals). The FITC-IgM solution was concentrated to 15 to 20 mg/mL, and aliquots were heat-aggregated in glass tubes at 63 °C for one-half hour with gentle agitation. After cooling on ice, the solution was diluted to 4.5 mL with PBS (pH 8.0), and the supernatant was ultracentrifuged at 145,000 g for one hour. The pelleted protein was resuspended in PBS. Working dilutions of aggregates contained approximately 2.5 mg/mL of protein and had OD_{495}/OD_{280} ratios between 1 and 2. The reagent was stable for four weeks, and all aggregate preparations were centrifuged at 1,700 g before use to remove larger insoluble aggregated material.

Surface Immunoglobulin Determination

Surface immunoglobulin (sIg) was determined by incubating 10^6 cells at 4 °C for one-half hour with 10 μL of the appropriate dilution of FITC-conjugated monospecific goat antisera to human gamma, alpha, mu, delta, kappa, and lambda chains and gammaglobulins, obtained from Meloy Laboratories (Springfield, Va) and Dakopatts (Copenhagen, Denmark). Since 1981, the $F(ab')_2$ portions of goat antibodies specific for human gamma, alpha, mu, delta, kappa, and lambda chains conjugated to FITC (Cappel Laboratories, Cochranville, Pa) were used in these studies. After incubation, the cells were washed three times in PBS, resuspended in glycerine-PBS on a glass slide, and a minimum of 200 cells was observed for membrane fluorescence under a Leitz Ortholux microscope with Opak-Fluor Vertical Ploem Illuminator (Ernst Leitz, Wetzlar, West Germany). Cells were counted and observed simultaneously with a phase-contrast condenser.

Cytoplasmic Mu Chain Determination

Cytoplasmic mu chain (cμ) was determined by staining methanol-fixed cells on a glass slide with a fluorescein-conjugated anti-mu chain reagent for one hour at 22 °C, after rehydration with PBS.

E Rosette and 19S EAC Rosette Determination

Spontaneous sheep erythrocyte rosettes (E) were assayed according to the method of Baxley et al.[7] The C'3 receptor was determined by 19S EAC rosetting, according to the method of Bianco et al.[8] A 19S EA reagent was always used as a control, and in those cases where the control was in excess of 5%, the test for C'3 receptor was considered invalid.

Monoclonal Antibody Characterization

The monoclonal antibodies used included: J5 (Coulter Immunology, Hialeah, Fla), detecting the common acute lymphoblastic leukemia antigen, CALLA; B1 (Coulter Immunology), detecting a 35,000-dalton B cell-associated surface antigen; Ia (New England Nuclear, Boston), detecting a multimeric surface complex of 33,000 and 28,000 daltons present on B cells, activated T cells, and macrophages; and Lyt-2 (New England Nuclear), detecting a 65,000- and 67,000-dalton complex found on virtually all peripheral T cells and thymocytes and cross-reacting with most CLL cells. Cells (10^6) were incubated with an appropriate dilution of the monoclonal antibody for one-half hour on ice and then washed twice. Fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories) of appropriate dilution (usually 1:5) was added to the cells, incubated on ice for one-half hour, and washed three times in PBS. The cells were resuspended in glycerine-PBS on a glass slide and were observed in the same manner as for surface immunoglobulin determination. Since 1982, studies were performed in parallel utilizing a Becton-Dickinson FACS Research Analyzer. Cells were resuspended in PBS, and 10^5 cells were analyzed on the FACS by exposure to mercury arc lamp epi-illumination at 488 nm, utilizing an FITC filter pack at a photomultiplier setting of 380 V.

TdT Determination

Cytoplasmic terminal deoxynucleotidyl transferase (TdT) was assessed by the method of Bollum,[9] using the assay kit supplied by Bethesda Research Laboratories (Gaithersburg, Md).

RESULTS

Of the 53 CLL cell populations analyzed between April 1977 and February 1983, 43 (81%) expressed restricted surface immunoglobulin, with the majority of these cases (35/43 or 81%) expressing sIgM ± sIgD. In all cases, the light chain on the surface was unequivocally monoclonal. The B cell Fc μ receptor (Fc μR) was present on 33 of 35 cases (94%) that bore sIgM. In 21 cases (64%), the Fc μR was present on a greater percentage of the cell population than was sIg, as detected by a polyvalent anti-Ig reagent (mean sIg^+ cells 66.4% ± 28.9% *v* Fc μR^+ cells 80.1% ± 15.2%) (see Table 1). In only 12 cases (36%) did the number of sIg^+ cells exceed the number of Fc μR^+ cells.

The cells of ten patients (19%) did not express any detectable surface immunoglobulin (sIg^-), nor did they form rosettes with sheep erythrocytes (E^-). As can be seen in Table 2, the number of lymphocytes staining with the polyvalent Ig antiserum ranged between < 1% and 7% in this sIg^- subset. Monospecific antiserum assessment of alpha, delta, gamma, and mu heavy chains and kappa and lambda light chains revealed percentages of stained cells of less than 5% in all cases, with the vast majority being less than 1% (data not shown). No light chain class restriction was noted for the small population of positive cells detected. The number of lymphocytes forming E rosettes varied from < 1% in several patients to 16% in one patient.

The cells of all ten cases of sIg^-E^- CLL were found to clearly react with the aggregated IgM reagent that detects the B cell Fc μ receptor. The percentage of Fc μR^+ cells varied from 16% to 96% of the cell popula-

Table 1. Surface Phenotypes of 53 CLL Patients

Monoclonal sIg	Total No. of Cases	No. Fc μR^+	Fc $\mu R^+ sIg^+$ Cases: Percent Fc μR^+ > Percent sIg^+	Fc $\mu R^+ sIg^+$ Cases: Percent Fc μR^+ < Percent sIg^+
IgM	19	17	10	7
IgM + IgD	16	16	11	5
IgD	5	4	3	1
IgG	3	2	0	2
sIg^-E^-	10	10	10	0
Totals	53	49	34	15

Table 2. Surface Phenotypes of Ten sIg⁻E⁻ CLL Cases*

Marker	Case No. 1	2	3	4	5	6	7	8	9	10
Latex phagocytosis	3	2	<1	2	1	10	1	<1	3	<1
E rosettes	<1	2	4	9	16	7	2	<1	11	11
7S Rosette	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
19S EAC rosette	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Polyvalent Ig	3	5	<1	<1	3	4	<1	<1	7	<1
Fc μR	80	40	70	72	40	16	75	70	80	63
Lyt-2	90	83	3	16	—	—	83	80	36	27
Ia	85	80	80	68	—	55	3	90	40	75
Cytoplasmic μ	(neg)	(neg)	neg	—	—	—	—	—	—	—
CALLA	<1	(1)	<1	<1	—	—	<1	<1	—	—
B1	(22)	(64)	8	6	—	—	25	20	—	—
TdT	neg	—	neg	—	—	—	—	neg	—	—

*Expressed as the percentage of positive cells in the population studied.
Parentheses indicate test results performed on later samples.
(—) Test not done.

tions (mean 64.2% ± 15.0%). In nine of the ten cases, greater than 40% of the cells were positive. The staining intensity for Fc μR^+ cells was characteristically graded 3+ to 4+ (defining a score of 1–2+ as a typical sIg^+ CLL cell's fluorescent intensity).

The sIg^-E^- cases were also evaluated for the presence of differentiation-related antigens with a panel of monoclonal antibodies. In the eight assessed for the presence of Ia-like antigen, Ia was expressed on all cell populations, with percentages of positive cells ranging from 40% to 92% (mean 73.1% ± 18.0%). The T-associated differentiation antigen that is also detected on sIg^+ CLL was sought in seven cases using the monoclonal antibody Lyt-2. Three of the seven cases initially had low percentages of cells positive for Lyt-2 (mean 4.7%) when compared with the Ia-like antigen (mean 65.0%) and/or Fc μR (mean 53.7%). Cells from six cases were stained for the presence of the CALLA antigen. Only one patient's cells expressed a small population of $CALLA^+$ cells (4%), while the Fc μR, Lyt-2, and Ia percentages were 70%, 80%, and 90%, respectively. The monoclonal antibody B1 reacted with six of six cell populations tested, but usually in low percentage. In only one case did a majority of the cells express B1 (64%). In the remaining five cases, between 6% and 25% of the cell population was positive for the B1-associated antigen.

All ten cases were assessed for the presence of the C'3 receptor, and in no instance was it detected. Three cell populations studied for detectable cytoplasmic mu chains or terminal deoxynucleotidyl transferase were negative.

Serial Surface Phenotype Determinations in sIg^-E^- Cases

Serial studies, done over as many as three years, were performed on several patients. Patient 1 (see Table 3) illustrates an instance where cells were initially negative for Fc μR yet acquired a high percentage of Fc μR^+ cells one year after initial phenotyping. Subsequent reassessment also showed a striking increase in the percentage of cells reacting with Lyt-2 (from 10% to 90% in 11 months). Ia^+ and Fc μR^+ cell populations have remained stable over this period. This patient received no therapy during this period, and there was no remarkable change in his clinical course during this time.

Patient 2 also showed phenotypic instability, as illustrated by the emergence of a $Lyt\text{-}2^+$ cell population. An Fc μR^+ cell population preceded the $Lyt\text{-}2^+$

Table 3. Serial Lymphocyte Surface Markers in Single Patients

	Patient 1				Patient 2			Patient 3	
	February 1980	March 1981	February 1982	April 1983	December 1980	March 1981	January 1982	January 1981	March 1983
E-rosettes	2*	<1	<1	<1	4	7	2	4	15
Polyvalent sIg	3	3	3	1	<1	<1	5	<1	68†
Fc μR	6	90	90	76	<1	90	40	70	96
Lyt-2	ND	10	90	40	<1	<1	83	3	48
Ia	ND	70	85	50	45	56	80	80	92

*Expressed as percentage of positive cells.
†λ Light chain.

population by ten months. This patient was being treated with alkylating agents throughout the time that his lymphocyte phenotypes were assessed.

Patient 3's cells also acquired increased reactivity with Lyt-2 over the course of two years. Reactivity for Ia and Fc μR was stable. The most striking change was the unequivocal appearance of restricted surface lambda light chain. Simultaneous heavy chain determinations could not be done at that time due to limited material available for study. This patient was also treated with alkylating agents in the interval.

DISCUSSION

The aggregated IgM reagent has been shown to react specifically with the IgM Fc receptor of B cells, without cross-reactivity with T cells.[6,10] In the majority of cases, the expression of aggregated IgM binding by CLL cells closely parallels the expression of surface immunoglobulin. As larger numbers of CLL cell populations have been studied, it has become apparent that a subset of CLL exists in which the Fcμ receptor, as detected by the aggregated IgM reagent, is expressed without surface immunoglobulin. A similar sIg^-E^-Fc μR^+ subset has been found in a series of our patients with acute lymphoblastic leukemia (ALL).[11] In the latter instance, the coexistence of CALLA, Ia-like antigen, and/or cytoplasmic mu chains has helped to confirm the B cell lineage of this ALL subset that bears the Fc mu receptor.

The sIg^-E^-Fc μR^+ CLL subset we describe here is also clearly of B cell origin, as all cases express the B cell Fc mu receptor (10/10 cases), Ia-like antigen (9/9 cases studied), and B1 antigen (6/6 cases studied). Ia-like antigen is uniformly found on the cells of B-CLL, but this marker is not specific[12-15] and requires corroborating evidence to define the cell as being of B cell lineage. B1 antigen is felt to be present exclusively on B cells[16] and was found to be uniformly expressed on the cells of the sIg^-E^-Fc μR^+ CLL subset, although the percentage of positive cells was much lower than the corresponding Ia- or Fc μR-positive percentage. This may reflect a lower density of B1 antigen at this early level of B cell maturation when compared to Ia-like antigen or the Fcμ receptor. Similar observations, relating increasing B1 intensity of expression to B cell maturation, have recently been made by Gordon and colleagues.[17]

Additional evidence for the B cell origin of one of our sIg^-E^- cases was seen in serial studies demonstrating the acquisition of a monoclonal surface light chain (patient 3). Although heavy chain determinations could not be performed, this case may illustrate an instance of CLL in which the maturational stage of the malignant lymphocyte is arrested at a stage similar to the six cases of $sIg^-E^-cIg^-$ CLL recently described by Hannam-Harris and associates.[18] In their cases, light immunoglobulin chains were found in the supernatant after 18 hours of in vitro tissue culture. Based on this observation, it was suggested that these cases may represent an arrest at an early stage of B cell maturation, which in the normal course of B cell development exhibits unbalanced light chain synthesis. Further evidence supporting this view was recently reported by Gordon and associates.[19]

The development of monoclonal antibodies detecting cell surface antigens has shed further light on the origin and maturational stage represented by cases of sIg^-E^- CLL. CLL cells usually express the 65,000-dalton membrane T cell-associated antigen complex, as detected by a variety of monoclonal antibodies, including Lyt-2, T101, and Leu-1. Royston et al and Dillman et al[20,21] studied the surface phenotypes of CLL patients with the monoclonal antibody T101 and found that the sIg^-E^- phenotype variably expressed the T65 antigen. In our series of sIg^-E^-Fc μR^+ cell populations, four of seven cases initially had a low percentage of Lyt-2^+ cells when compared to Ia^+ or Fc μR^+ cell percentages. As shown in Table 3, in three of these cases, serial studies showed that the percentage of Lyt-2^+ cells had increased significantly. Thus, the 65,000-dalton antigen, which Lyt-2 and T101 detect, may first appear near the stage of B cell differentiation defined by the appearance of the Fcμ receptor, as all of our cases, regardless of Lyt-2 status, already expressed Fc μR.

The apparent close association between the expression of Fc μR and T65 antigen is further supported by the recent report of Schroff and colleagues,[22] who described three cases of CLL with the phenotype $sIg^-E^-cIg^+$Leu-1^+. These cases relate the appearance of the T cell-related antigen (as defined by Leu-1) to the stage of B cell development at which the cytoplasmic mu chain is still detectable. Although none of our sIg^-E^- CLL patients exhibited an overlap pre-B phenotype (cytoplasmic mu and Fcμ receptor coexpressed), we have recently described one patient with ALL with just such a phenotype: $E^-sIg^-c\mu^+$Fc μR^+.[11] Our serial studies in several patients illustrate that cells with the sIg^-E^- Fc μR^+ phenotype may not initially express the T65 antigen, but it can be acquired later in the course of the patient's disease. Thus, Fcμ receptor, in contrast to the T65 antigen, seems to be expressed uniformly at this early differentiation stage and continues to be expressed even as surface IgM appears at a more mature B cell stage.

Current concepts of B cell differentiation define a pre-B cell ($sIg^-c\mu^+$) as a discrete maturational stage preceding the appearance of cells bearing sIgM (early

B cells). Our data suggest that CLL cells with the phenotype $sIg^-E^-c\mu^-$ Fc μR^+ represent clonal expansions of B cells in transit between these two stages. The variable expression of CALLA, B1, and the T65 antigen on the cell surface of this subset lends further support to this view. It should be emphasized, however, that a variable degree of in vivo and in vitro clonal instability exists with respect to the expression of markers, as evidenced by our serial studies and those of others. Taken in this context, we have found that the B cell Fcμ receptor has been a highly useful and consistent marker for defining this early sIg^-E^- ("null") CLL phenotype.

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