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

By Richard A. Rueders and Peeter A. Poldre

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 (slg⁺) 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 slg 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 slgM⁺ early B cell. The B cell Fc μR appears to be a consistent and potentially useful marker for slg E⁻ (“null”) CLL.

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. 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 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. 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 
OD495/OD280 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 (slg) was determined by incubating 10⁶ 
cells at 4 °C for one-half hour with 10 μL of the appropriate dilution 
of FITC-conjugated monospecific goat antibodies specific 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, Cochran- 
ville, 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 after staining with the 
polyvalent anti-Ig reagent (mean slg). In 21 cases (64%), the Fe slgR was present on 33 of 35 cases (94%) that bore 
slgM. In 21 cases (64%), the Fe slgR was present on a 
greater percentage of the cell population than was slg, 
as detected by a polyclonal anti-Ig reagent (mean slg⁺ 
cells 66.4% ± 28.9% vs. Fe slg⁻ cells 80.1% ± 15.2%) (see Table 1). In only 12 cases (36%) did the number of 
slg⁺ cells exceed the number of Fe slg⁻ cells. 

Cytoplasmic Mu Chain Determination 
Cytoplasmic mu chain (μ) 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. The C3 receptor was determined by 
19S EAC rosetting, according to the method of Bianco et al. 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 C3 receptor was 
considered invalid.

Monoclonal Antibody Characterization 
The monoclonal antibodies used included: J5 (Coulter Immunol- 
onometry, Hialeah, Fla), detecting the common acute lymphoblastic leuke-

<table>
<thead>
<tr>
<th>Table 1. Surface Phenotypes of 53 CLL Patients</th>
<th>FctR slg⁺ Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal slg</td>
<td>Total No. of Cases</td>
</tr>
<tr>
<td>IgM</td>
<td>19</td>
</tr>
<tr>
<td>IgM + IgD</td>
<td>16</td>
</tr>
<tr>
<td>IgD</td>
<td>5</td>
</tr>
<tr>
<td>IgG</td>
<td>3</td>
</tr>
<tr>
<td>slg 'E'</td>
<td>10</td>
</tr>
<tr>
<td>Totals</td>
<td>53</td>
</tr>
</tbody>
</table>

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 slgM ± slgD. 
In all cases, the light chain on the surface was un-
equivocally monoclonal. The B cell Fe μ receptor (Fc μR) was present on 33 of 35 cases (94%) that bore 
slgM. In 21 cases (64%), the Fe μR was present on a 
greater percentage of the cell population than was slg, 
as detected by a polyclonal anti-Ig reagent (mean slg + 
cells 66.4% ± 28.9% vs. Fe μR⁻ cells 80.1% ± 15.2%) (see Table 1). In only 12 cases (36%) did the number of 
slg⁺ cells exceed the number of Fe μR⁻ cells. 

The cells of ten patients (19%) did not express any 
detectable surface immunoglobulin (slg⁻), 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 slg⁻ subset. Monospe-
cific 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 slg 'E' CLL were found 
to clearly react with the aggregated IgM reagent that 
detects the B cell Fe μ receptor. The percentage of Fe 
μR⁺ cells varied from 16% to 96% of the cell popula-
THE Fc\textsubscript{R} RECEPTOR IN sIg\textsuperscript{E} \textsuperscript{-} CLL

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Expressed as percentage of positive cells.

Table 2. Surface Phenotypes of Ten sIg\textsuperscript{E} \textsuperscript{-} CLL Cases*

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

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

Serial Surface Phenotype Determinations in sIg\textsuperscript{E} \textsuperscript{-} CLL 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+ cells one year after initial phenotyping. Subsequent reassessment also showed a striking increase in the percentage of cells expressing Fc µR+ (mean 45.7%) 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 C3 receptor, and in no instance was it detected. Three cell populations studied for detectable cytoplasmic mu chains or terminal deoxynucleotidyl transferase were negative.

Table 3. Serial Lymphocyte Surface Markers in Single Patients

<table>
<thead>
<tr>
<th>Patient 1</th>
<th></th>
<th></th>
<th></th>
<th>Patient 2</th>
<th></th>
<th></th>
<th></th>
<th>Patient 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>February</td>
<td>March</td>
<td>January</td>
<td>December</td>
<td>March</td>
<td>January</td>
<td>December</td>
<td>March</td>
<td>January</td>
<td></td>
</tr>
<tr>
<td>E-rosettes</td>
<td>2*</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Polyvalent sIg</td>
<td>3 3 3 1</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td>90 90 76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fc µR</td>
<td>6</td>
<td>90</td>
<td>90 76</td>
<td>&lt;1</td>
<td>&lt;1 5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>68†</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Lyt-2</td>
<td>ND</td>
<td>10</td>
<td>10 40</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>83</td>
<td>3</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>ND</td>
<td>70</td>
<td>85 50</td>
<td>45</td>
<td>56</td>
<td>80</td>
<td>80</td>
<td>92</td>
<td></td>
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</tr>
</tbody>
</table>

*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 \( \mu R \) 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. 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 FcR receptor, as detected by the aggregated IgM reagent, is expressed without surface immunoglobulin. A similar slgE \( E^-Fc \mu R^- \) subset has been found in a series of our patients with acute lymphoblastic leukemia (ALL). 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 slgE \( E^-Fc \mu R^- \) 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 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 and was found to be uniformly expressed on the cells of the slgE \( E^-Fc \mu R^- \) CLL subset, although the percentage of positive cells was much lower than the corresponding Ia- or Fc \( \mu R^- \) 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 Fc\( \mu \) receptor. Similar observations, relating increasing B1 intensity of expression to B cell maturation, have recently been made by Gordon and colleagues.

Additional evidence for the B cell origin of one of our slgE \( E^- \) 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 slgE \( E^-clg^- \) CLL recently described by Hannam-Harris and associates. 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 maturational stage, 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.

The development of monoclonal antibodies detecting cell surface antigens has shed further light on the origin and maturational stage represented by cases of slgE \( E^- \) 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 studied the surface phenotypes of CLL patients with the monoclonal antibody T101 and found that the slgE \( E^- \) phenotype variably expressed the T65 antigen. In our series of slgE \( E^-Fc \mu R^- \) cell populations, four of seven cases initially had a low percentage of Lyt-2-positive cells when compared to Ia- or Fc \( \mu R^- \) 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 Fc\( \mu \) receptor, as all of our cases, regardless of Lyt-2 status, already expressed Fc \( \mu R^- \).

The apparent close association between the expression of Fc \( \mu R^- \) and T65 antigen is further supported by the recent report of Schroff and colleagues, who described three cases of CLL with the phenotype slgE \( E^-clg^-Leu-1^- \). 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 slgE \( E^- \) CLL patients exhibited an overlap pre-B phenotype (cytoplasmic mu and Fc\( \mu \) receptor coexpressed), we have recently described one patient with ALL with just such a phenotype: E slgE \( clg^- \ mu Fc \mu R^- \). Our serial studies in several patients illustrate that cells with the slgE \( E^-Fc \mu R^- \) phenotype may not initially express the T65 antigen, but it can be acquired later in the course of the patient’s disease. Thus, Fc\( \mu \) 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 (slg\( clg^- \)) as a discrete maturational stage preceding the appearance of cells bearing slgM (early
B cells). Our data suggest that CLL cells with the phenotype sIgE^E^c^F^C^R 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 FcR receptor has been a highly useful and consistent marker for defining this early sIgE^E^ (“null”) CLL phenotype.

ACKNOWLEDGMENT

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