Chronic Lymphocytic Leukemia Progenitor Cells Carry the Antigens T65, BA-1, and Ia

By Robert T. Perri, Ivor Royston, Tucker W. LeBien, and Neil E. Kay

CLL B cells may be induced to form B-cell colonies in vitro. Colonies formed are monoclonal and appear to reflect the circulating malignant B-cell clone in vitro. Using hybridoma-produced monoclonal antibodies (MAB) and an in vitro B-cell colony assay, we have provided a characterization of the antigenic phenotype of the clonogenic CLL B cell. B-cell colony growth in both patients and normals was not altered by prior incubation with either MAB or complement (C') alone. CLL B-cell colony formation was markedly reduced after treatment with T101 and C', while normal colonies were unaffected (8 ± 2 versus 107 ± 10). None of the residual CLL B-cell colonies after T-101 and C' treatment reacted with T-101. However, BA-1 and Ia reactivity were still seen in residual CLL B-cell colonies following T-101 treatment. In contrast, a similar percentage reduction of B-cell colony growth was seen for both normals and CLL patients following treatment with BA-1 (76% versus 81%) and Q5/13 (89% versus 92%). These studies suggest that the CLL progenitor cell is characterized by the phenotype Ia', BA-1', T-101'. Better definition of the CLL progenitor cell has potential implications with regards to clinical utilization of MAB in the treatment of CLL.

CHRONIC LYMPHOCYTIC leukemia (CLL) is characterized by the accumulation of small and uniform-appearing lymphocytes which, in most instances, are proliferating monoclonal B cells.1,2 Previous attempts to define CLL B cells have noted the following differences between malignant CLL and normal B cells: the presence of low density surface immunoglobulin (SIg),3 a decreased capability to redistribute SIg rapidly at 37°C to form bright caps when stained with fluorescein-labeled anti-immunoglobulin (lg),3 and a higher percentage of cells forming rosettes with mouse red blood cells (MRBC).4,5 The formation of MRBC rosettes, which has been considered by some investigators to represent a marker of early B cells, has led to the conclusion that CLL B cells may be derived from a clone of cells arrested at an early stage of B-cell differentiation.2,6,7

The murine hybridoma system8 has now made available a variety of monoclonal antibodies that provides an opportunity to study the maturation arrest of CLL. Such investigations provide further phenotypic characterization of the malignant CLL B cell. Several monoclonal antibodies have been produced that share reactivity with cell surface determinants present on SIg+ CLL cells. Abramson et al. have produced a monoclonal antibody, BA-1, against the pre-B acute lymphoblastic leukemia cell line, NALM-6-M1.9 BA-1 identifies a cell surface determinant primarily expressed on normal and malignant B cells that is lost when these cells terminally differentiate into plasma cells. BA-1 reacts with all SIg+ CLL and >80% of SIg+ non-Hodgkin’s lymphomas. Royston et al. have produced a monoclonal antibody, T-101, which identifies a 65,000-dalton cell surface determinant present on normal and malignant human T lymphocytes and SIg+ CLL cells, but not on normal SIg- cells.10 T-101 reactivity on SIg+ CLL cells appears restricted to “immature” nonsecretory cells. More “mature” cases of SIg+ CLL associated with a circulating M-protein are T-101-. Cytofluorographic analysis revealed the T65 antigen density to be lower on SIg+ CLL cells compared with normal peripheral blood T cells.11 Complement-dependent cytotoxicity studies using T-101 have demonstrated cytotoxicity for normal T colony-forming cells but not for granulocyte-macrophage (CFU-C) and erythroid (BFU-E) progenitors.12

We have recently reported on the utility of an in vitro B-cell colony assay system for evaluating B-cell growth in patients with CLL.13 CLL colonies formed were shown to be monoclonal and appeared to reflect the circulating malignant B-cell clone in vitro. Using the above-mentioned hybridoma-produced monoclonal antibodies and in vitro B-cell colony assay, we examined normal and CLL B-cell colonies. These studies begin to provide a characterization of the antigenic phenotype of the clonogenic CLL B cell.

MATERIALS AND METHODS

Six patients with CLL and six age and sex-matched volunteers were studied. No patient had received any therapy for at least 6 wk prior to being studied. All patients were staged by the clinical...
staging system proposed by Rai. Peripheral blood mononuclear cells were isolated from venous blood by Ficoll/Hypaque (F/H). Monocyte depletion was done by adherence in tissue culture flasks for 45 min at 37°C in 5% CO₂, and the nonadherent cells were harvested. Nonadherent cells were then allowed to rosette with AET-sensitized sheep erythrocytes (SRBC) at 4°C for 2 hr. Rosetted T cells were separated from the nonrosoted B and null cells (hereafter called B cells) by two successive F/H gradients, and the SRBC were removed by hypotonic lysis with distilled water. T lymphocytes were then irradiated with 3000 rads.

PHA-T-cell conditioned medium (PHA-TCM) was prepared by incubating T cells with 1% PHA in α-MEM with 10% FCS (GIBCO, Grand Island, NY) at 37°C with 5% CO₂ for 3 days. Supernatants were then collected, filtered, and stored at 4°C.

The B-cell colony assay was performed as previously described. In brief, B cells were suspended to 2 x 10⁵/ml in α-MEM with 10% FCS, 5% CO₂ for 2 hr. Rosetted T cells were separated from the nonrosoted B and null cells (hereafter called B cells) by two successive F/H gradients, and the SRBC were removed by hypotonic lysis with distilled water. T lymphocytes were then irradiated with 3000 rads.

PHA-T-cell conditioned medium (PHA-TCM) was prepared by incubating T cells with 1% PHA in α-MEM with 10% FCS (GIBCO, Grand Island, NY) at 37°C with 5% CO₂ for 3 days. Supernatants were then collected, filtered, and stored at 4°C.

The B-cell colony assay was performed as previously described. In brief, B cells were suspended to 2 x 10⁵/ml in the presence of 3 x 10⁵/ml irradiated T cells in α-MEM with 10% FCS, 20% PHA-TCM, and 0.8% methylcellulose. After vortexing, 0.1-ml aliquots were placed into 6-mm flat-bottomed microtiter wells (Linbro, Hamden, CT). All experiments were done in triplicate. Plates were secured tightly and incubated at 37°C for 5–7 days.

Colonies numbers were quantified after 5 days of in vitro growth by counting the number of colonies present in each microtiter well using an Olympus inverted microscope. A colony was defined as a distinct aggregate of greater than 20 cells.

Inhibition of B-cell colony formation was assessed by incubating 10⁷/ml peripheral blood normal or CLL B cells in α-MEM with varying dilutions (1:10,1:100,1:1,000) of T-101, BA-1, an Irreactive monoclonal antibody Q5/13, control ascites, and/or complement for 1 hr at 37°C. Indirect immunofluorescence was used to assess binding of monoclonal antibody to peripheral blood B cells. Dilutions of antibody previously shown to result in optimal antibody–B-cell binding were used as described. Prescreened rabbit complement (Pelfreez, Rogers, AK) was used at a final concentration of 10%. The percentage colony growth was determined on day 5 by comparing the growth of B-cell colonies after this preincubation with antibody and/or complement to that of controls incubated in media alone.

The following are some of the identifying characteristics of the monoclonal antibodies that were used: (1) T-101 is an IgG₂ antibody that recognizes a 65,000-dalton T-cell-associated antigen also expressed on Slg⁺ B cells. (2) BA-1 is an IgM antibody that recognizes an antigen present on the vast majority of normal and malignant Slg⁺ cells, which is lost as B cells mature into plasma cells. (3) Q5/13 is an IgG₁ antibody that recognizes a nonpolymorphic HLA-DR determinant. This antibody was kindly provided by Dr. S. Ferrone, Columbia University, New York, NY. All 3 monoclonal antibodies effectively fix complement.

Membrane Analysis of Colony Cells

Pooled colony cells from ≥50 colonies were washed 3 times in PBS with 2% BSA and 0.2% sodium azide. Cells were suspended in α-MEM to 2 x 10⁶/ml, and 0.05 ml of this pooled colony cell suspension was mixed with 0.05 ml of a 1:100 dilution of murine monoclonal antibody. Antibodies used included T-101, BA-1, and Q5/13. The suspension of pooled colony cells and monoclonal antibody was incubated for 45 min in an ice bath. Cells were then washed 3 times in cold α-MEM and resuspended in 0.1 ml α-MEM. This suspension was incubated for 45 min in an ice bath. Cells were then washed 3 times in cold α-MEM, resuspended in a minimal volume, and observed immediately for immunofluorescence.

Statistical Analysis

Results are reported as mean number of colonies formed from triplicate wells ± SEM. All statistical analyses were performed with the Student’s t test.

RESULTS

When peripheral blood B cells were not exposed to monoclonal antibodies, the number of B-cell colonies formed was significantly less in patients than in normals (p < 0.001) (Table 1). B-cell colony growth in both normals and CLL patients was not reduced by prior incubation of B cells with the murine monoclonal antibodies BA-1, T-101, Q5/13, or control ascites alone (Table 1). No significant effect on B-cell colony growth occurred when B cells were incubated with complement alone.

Preincubation of peripheral blood B cells with the monoclonal antibody Q5/13 and complement resulted in a significant reduction in both normal and CLL B-cell colony growth. A similar percentage reduction of B-cell colony growth was seen for both normals and CLL patients (89% versus 92%) (Table 2).

Preincubation of peripheral blood B cells with the monoclonal antibody BA-1 and complement resulted in a significant reduction in both normal and CLL B-cell colony growth. The percentage reduction of B-cell colony growth was similar for both normals and CLL patients (76% versus 81%) (Table 2).

However, following exposure to T-101 and complement, CLL B-cell colony formation was markedly reduced (87%), while normal B-cell colony formation was essentially unaffected (3%) (p < 0.001).

Residual CLL B-cell colonies after T-101 and complement exposure were examined for possible persistent reactivity with BA-1, T-101, or Q5/13. None of

| Table 1. Normal and CLL B-Cell Colony Growth After Antibody or C' Treatment Alone |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| None                           | Q5/13           | BA-1            | T-101           | C'              | Control Ascites |
| (n = 6)                        |                 |                 |                 |                 |                 |
| Normals                        | 127 ± 8*        | 120 ± 8         | 127 ± 13        | 110 ± 15        | 116 ± 13        | 122 ± 10        |
| (n = 6)                        | 66 ± 18         | 63 ± 23         | 69 ± 20         | 62 ± 23         | 68 ± 18         | 67 ± 16         |

*Numbers represent mean number of colonies formed from triplicate wells ± SEM. Colony numbers are determined on day 5, as described in Materials and Methods.
the residual CLL B-cell colonies following T-101 exposure reacted with T-101. However, Ia and BA-1 activity were still seen in residual CLL B-cell colonies after T-101 exposure (Table 3). When residual CLL B-cell colonies were studied following Q5/13 or BA-1 exposure, T-101 reactivity was not seen in persistent colony cells (data not shown).

**DISCUSSION**

Although it has been recognized for many years that CLL is a malignant lymphoproliferative disorder, usually of B-cell origin, numerous studies have failed to clearly establish the relationship of the malignant CLL B cell to normal B-cell development and differentiation. We recently reported on an in vitro B-cell colony assay system for evaluating the circulating malignant B-cell clone in CLL.\(^{13}\) Using this in vitro B-cell colony assay system and monoclonal antibodies BA-1, T-101, and Q5/13, we attempted to define further the phenotypic characteristics of normal and CLL B-cell colony progenitor cells.

Previous studies showing the presence of SIg have confirmed the B-cell origin of these colonies.\(^{13,16}\) CLL colonies were also shown to be monoclonal and appeared to reflect the circulating malignant clone in vitro. Studies presented here are consistent with a B-cell lineage for both normal and CLL colonies. HLA-DR (Ia-like antigen), a glycosylated 2-chain polypeptide coded for by the HLA-D/DR locus, is known to be present on the majority of lymphoid cells of B-cell origin.\(^{18}\) Most SIg\(^+\) B cells are also Ia\(^+\). During the terminal stages of B-cell differentiation, Ia antigens are lost.\(^{18}\) The HLA-DR\(^+\) cells found in CLL colonies are consistent with the previously reported observation that Wright-Giemsa staining of cells from single CLL colonies failed to show evidence of typical plasma cells.\(^{13}\) Thus, terminal B-cell differentiation in vitro does not appear to occur in CLL B-cell colonies.

Using NALM-6-Mi, a pre-B (clg\(^+\), SIg\(^-\)) acute lymphoblastic leukemia cell line,\(^{9}\) a monoclonal antibody designated BA-1 was produced.\(^{8}\) BA-1 recognizes a determinant primarily expressed on normal B cells, which is lost when these cells terminally differentiate into plasma cells. This determinant has also been noted to be present on the malignant CLL B lymphocyte. Our findings confirm the presence of BA-1 reactivity in both normal and CLL B-cell colony progenitor cells. The BA-1\(^+\) in CLL colony cells is also consistent with the previously reported observation of lack of differentiation into mature plasma cells in CLL colonies.\(^{13}\)

T-101 was the only monoclonal antibody that appeared to recognize a surface determinant present on CLL B-cell colony progenitor cells but absent from normal B-cell colony progenitor cells. T-101 identifies a 65,000-dalton cell surface determinant present on normal and malignant human T lymphocytes but not on normal SIg\(^+\) cells.\(^{10,12}\) It has been shown to be present on normal T cell- and B-cell colonies but absent on normal granulocyte-macrophage (CFU-C) and erythroid (BFU-E) progenitors.\(^{12}\) Previous studies demonstrating the presence of receptors for the lectin *Helix pomatia* (which is normally found only on T cells) have suggested the presence of dual T-cell and B-cell markers in some patients with CLL.\(^{21}\) Prior studies with heteroantiserum against human thymus and T cells, which were adsorbed with cells other than CLL, also have demonstrated shared reactivity between normal T cells and SIg\(^+\) CLL cells but not normal SIg\(^-\) cells.\(^{22,23}\) Royston et al.\(^{16}\) have demonstrated T-101 reactivity with peripheral blood SIg\(^+\) CLL cells. Our findings in this study demonstrate the presence of T-101-reactive CLL B-cell colony progenitor cells. Recently, Janossy et al.\(^{24}\) have identified an infrequent (2%-3%) B-lymphocyte subpopulation present in normal human tonsil and lymph nodes whose phenotype includes a similar 65,000-dalton cell surface determinant. The malignant CLL B lymphocyte may arise from this normal B-lymphocyte counterpart.

It appears that the cell responsible for in vitro B-cell colony formation in CLL has a determinant reactive with T-101. The cell responsible for normal B-cell colony formation in vitro does not appear to share such a determinant. Both the normal and CLL progenitor cells for B-cell colony formation in vitro appear to share Ia and BA-1 reactivity.

These findings are supportive of the hypothesis that CLL represents a lymphoproliferative disorder "arrested" in an early stage of B-lymphocyte development.

### Table 2. Inhibition of Normal and CLL B-Cell Colony Growth by Antibody Plus C' Treatment

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal (n = 6)</th>
<th>CLL (n = 6)</th>
<th>Normal (n = 6)</th>
<th>CLL (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5/13 plus C'</td>
<td>13 ± 6(^{\star})</td>
<td>99%</td>
<td>4 ± 2</td>
<td>93%</td>
</tr>
<tr>
<td>BA-1 plus C'</td>
<td>30 ± 8</td>
<td>76%</td>
<td>13 ± 7</td>
<td>81%</td>
</tr>
<tr>
<td>T-101 plus C'</td>
<td>107 ± 10</td>
<td>3%</td>
<td>8 ± 2</td>
<td>87%</td>
</tr>
</tbody>
</table>

\(^{\star}\)Numbers represent mean number of colonies formed from triplicate wells ± SEM. Colony numbers are determined on day 5, as described in Materials and Methods.

\(\Delta\)Percent reduction of B-cell colony growth after exposure to antibody plus C' from B-cell colony growth after exposure to antibody alone.

### Table 3. Effect of T-101 and Complement Treatment on Residual CLL Colony Cell Phenotype

<table>
<thead>
<tr>
<th>Monoclonal Antibody Studied</th>
<th>Percent Reactive Colony Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-101</td>
<td>0%</td>
</tr>
<tr>
<td>BA-1</td>
<td>80%</td>
</tr>
<tr>
<td>Q5/13</td>
<td>91%</td>
</tr>
</tbody>
</table>

\(\star\)Percent positive cells with monoclonal antibody indicated determined as described in Materials and Methods (200 cells counted).
and differentiation, as postulated by other investigators.2,6,7,25 Tottman's use of phorbol esters to induce terminal differentiation of malignant SIg⁺ CLL cells into mature plasma cells appears to support this concept of "arrested" B-cell development in CLL.28

On the basis of these studies, a presumed localization of CLL in the scheme of B-cell differentiation and development may be distinguished. The CLL progenitor cell appears to be a cell arrested in its development at an "immature" stage characterized by the phenotype Ia⁺, BA-i⁺, T-101⁺. With this in mind, we would postulate CLL's location in a scheme of B-cell differentiation as shown in Fig. 1. SIg⁺ CLL appears to represent a proliferative disorder of B cells intermediate in differentiation between the small pre-B-cells and the SIgM⁺, SIgD⁺ B cells. It remains for future studies to clarify if the CLL progenitor cell is more or less differentiated than the SIgM⁺, SIgD⁺ B cell.

In summary, our findings demonstrate that CLL B-cell colony formation in vitro does reflect the malignant clone in vivo. This assay provides a means for further evaluating the relationship of the malignant B-cell to normal B-cell development and differentiation. Using this assay, we have been able to further define the phenotypic characteristics of the CLL B-cell colony-forming cells and have suggested their possible relationship to normal B-cell development. Better definition of the CLL progenitor cell has potential implications with regards to clinical utilization of monoclonal antibodies in the treatment of CLL.27

REFERENCES

20. Royston I, Majda JA, Yamamoto GY, Baild SM: Monoclonal antibody specific for normal and neoplastic human T cells, in


Chronic lymphocytic leukemia progenitor cells carry the antigens T65, BA-1, and la

RT Perri, I Royston, TW LeBien and NE Kay