Subpopulation Heterogeneity in Human Acute Myeloid Leukemia Determined by Monoclonal Antibodies

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The leukemic population in 63 patients with acute myeloid leukemia (AML) was studied with 15 monoclonal antibodies that detect lineage-related and stage-related antigens on normal hemopoietic cells. Indirect immunofluorescence and fluorescence-activated cell sorting showed that subpopulations of leukemic cells reacted with some or all antibodies, but the percentage of cells reacting with a single antibody varied widely among patients. The composite antigenic phenotype of the various cases, as determined by immunofluorescence assay, did not correlate with the French-American-British morphological classification. Furthermore, some cells in each case failed to express any antigen normally expressed on myelomonocytic precursors from the level of the early CFU-GM to the mature granulocyte or monocyte. In double-fluorescence experiments, the individual cells expressed none, one, or both antigens. These results demonstrate that there is considerable subpopulation heterogeneity in AML. This heterogeneity may considerably limit or complicate the use of monoclonal antibodies for diagnosis, prognosis, and treatment of acute nonlymphocytic leukemia (ANLL).

Past investigations of the potential application of monoclonal antibodies in diagnosis and/or therapy of leukemia have focused on classifying leukemias as either lymphoid or myeloid and on identifying leukemia-specific antigens. Although the search for the latter has not been successful, monoclonal antibodies that react with lineage-related antigens, such as the common acute lymphocytic leukemia (CALLA) or granulocyte-monocyte-related antigens have been applied in the diagnosis and prognosis of acute and chronic leukemia. Most reports classify a leukemia as antigen-positive if ≥20% of the cells are positive for fluorescence when tested with a particular monoclonal antibody. Although this arbitrary criterion of classification is convenient and reproducible, it fails to account for the substantial number of leukemic cells that do not express the lineage-related antigens. The coexistence within a given leukemic population of antigen-negative and antigen-positive cells suggests that considerable subpopulation antigenic heterogeneity exists.

In the present study, we analyzed leukemic blasts from patients with acute myeloid leukemia (AML) by indirect immunofluorescence for reactivity with a panel of lineage-related and stage-related monoclonal antibodies that react with normal hemopoietic cells. The composite phenotype of the leukemic population was determined and correlated with the French-American-British (FAB) classification. Five cases were studied at the time of diagnosis, during remission, and at relapse. Finally, the coexpression of two antigens on single cells was determined by double-fluorescence labeling.

Materials and Methods

Patients and Normal Controls

The 63 patients with AML included 20 pediatric patients, aged 4 months to 16 years, from the Children's Hospital of Philadelphia, 17 adult patients from Hahnemann Medical College and Hospital, and 26 adult patients from the Ospedale S. Giovanni of Torino, Italy. Diagnostic marrow aspirates (36 cases) and/or peripheral blood (27 cases) from patients in whom blast replacement was more than 70% were obtained prior to therapy, at the time of diagnosis, and/or at the time of relapse. Diagnosis of AML was based on clinical, morphological, and histochemical criteria according to the FAB classification.

Normal blood and marrow cells were obtained from adult volunteers and from surveillance marrows of patients with solid tumors or pediatric patients with nonmalignant disease. These studies were approved by the Committees for Protection of Human Subjects at the three institutions involved.

Preparation or Separation of Cells

Blood or marrow samples were collected into tubes or syringes containing preservative-free heparin. Buffy coat cells from blood were separated from the upper two-thirds of the plasma by Plasmagel (30%, vol/vol; Roger Bellou Lab, Nevelly, France) sedimentation of erythrocytes at 37°C for 45 minutes; contaminating erythrocytes were lysed with hypotonic medium. Mononuclear cells were prepared by Ficoll-Paque gradient centrifugation (Pharmacia, Piscataway, NJ) (1.077 g/mL), and monocytes were isolated by double adherence (45 minutes at 37°C) in Petri culture dishes. Nonadherent cells were incubated with neuraminidase-treated sheep erythrocytes for 30 minutes on ice and then centrifuged on a Ficoll-Paque gradient. E rosette-forming cells were recovered from the pellet and E rosette-negative cells from the interface. Granulocytes were prepared from the Pellet obtained after centrifugation of theuffy coat cells on Ficoll-Paque gradient.

From the Wistar Institute of Anatomy and Biology, the Hahnemann Medical School, and the Children's Hospital of Philadelphia.

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Monoclonal Antibodies

Several of the monoclonal antibodies directed against surface differentiation antigens used in this study have been described previously.3-10,16-20 Antibodies SK37.7 and S1-19-9 (anti-HLA-DR) were gifts of Drs Koprowski and Trucco of the Wistar Institute.11,17 All other antibodies were generated in our laboratory by immunizing BALB/c mice with five different myeloid cell lines (KG1, ML3, HL60, BV173, K562), three primary leukemias (one M1, one M3, and one M4), and an avian myeloblastosis virus (AMV) transformed avian leukemia cell line.

Antibody-producing clones were selected on the basis of activity as titrated against suitable leukemic cell lines and peripheral blood leukocytes. Antibodies were used as ascites containing 2-6 mg of immunoglobulin/mL. A titer (usually 1:100 or 1:1,000 dilution of ascites) at which reactivity with target cells was consistently >90% was used in the studies reported here. The antigens detected by the antibodies were determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis.11,12,22

Immunofluorescence, Flow Cytofluorimetric Analysis, and Cell-Sorting Experiments

For indirect immunofluorescence, target cells were incubated for 30 minutes at room temperature with an appropriate dilution of antibody. After 3 rinses in phosphate-buffered saline (PBS), cells were incubated for 30 minutes at 4°C with a FITC-conjugated goat anti-mouse antibody (Cappel, Cochranville, Pa) preadsorbed with human immunoglobulins. Control cells were treated with ascites of parental myeloma cell line and the FITC-conjugated antibody. An Ortho Cytofluorograf System 50HH, connected to a Data General MP/200 microprocessor (Ortho Instruments, Westwood, Mass) was used for flow cytometric analysis. Threshold fluorescence intensity was selected at which 99% of the total cell population treated with control ascites of the parental myeloma and the FITC-conjugated antibody were negative. For each antibody, 3 x 106 cells/sample were analyzed; granulocytes, monocytes, and lymphocytes were distinguished on the basis of their right and forward angle scatter characteristics, and fluorescence intensity was determined over 200 or 1,000 channels simultaneously for the three different cell types.

To study simultaneous expression of two surface markers, normal cells and cells from several cases of leukemia were analyzed by fluorescence double-labeling. Leukemic cells were incubated with biotinylated antibodies followed by Avidin Texas Red.21 After three washes in cold PBS, cells were incubated with fluorescent-labeled antibody, and green and red fluorescent populations were compared.

For sorting experiments, a minimum of 6 x 106 cells/sample were used, and after indirect immunofluorescence labeling, they were resuspended into 1 mL of PBS. Sorting rate was 3 x 103 cell/s, with an anticoincidence setting of 3. Positive and negative cells were collected separately into tubes containing 1 mL of PBS supplemented with 10% fetal bovine serum (FBS). Cytocentrifuge slides were stained either with Wright-Giemsa or with May-Grünwald-Giemsa. At least 500 cells/sample were identified morphologically, and at least 3 separate sorting experiments on different bone marrow were performed with each monoclonal antibody tested.

Myeloid Progenitor Cell Reactivity

Complement-dependent cytotoxicity assay and cell sorting were used, respectively, to determine the reactivity of normal CFU-GM with cytotoxic and noncytotoxic monoclonal antibodies. For the complement-dependent cytotoxicity test, 4 x 105 marrow or 4 x 106 peripheral blood low density (<1.077 g/L) nonadherent cells for each sample were incubated in 0.2 mL of Iscove’s modified Dulbecco’s medium (IMDM), containing 10% FBS, with an equal volume of monoclonal antibody at a concentration able to saturate all antigenic determinants in predetermined target cells. After a 40-minute incubation at 4°C, 0.4 mL of absorbed rabbit complement (Low Tox H, Accurate Chemical Co) diluted 1:4 was added (final dilution 1:8) and the cells incubated for an additional 90 minutes at 37°C. Three control samples were included in each experiment: (1) cells incubated with medium plus FBS during the 2 incubations; (2) cells incubated with medium during the first incubation and then with complement alone; (3) cells incubated for 130 minutes with antibodies without complement. After the second incubation, cells were washed and plated in agar culture. The reactivity of antibodies to CFU-GM was expressed as percentage of inhibition of colonies in cultures of cells treated with antibodies and complement compared to control cultures.

CFU-GM reactivity with noncytotoxic monoclonal antibodies was tested in indirect immunofluorescence and cell sorting as described for the morphological analysis, except that sterile conditions were maintained. Fluorescent and nonfluorescent cells were separately collected and plated in agar culture. Reactivity was expressed as the percentage of total CFU-GM present in the unfractionated population, which was found among the fluorescent cells.

Marrow and peripheral blood cells were cultured in Petri dishes in IMDM containing 20% FBS, 10% giant-cell tumor (GCT)-conditioned medium,24 and 0.3% agar (DIFCO, Detroit, Mich); 106 bone marrow or 107 peripheral blood cells were plated in each dish and 3 dishes were plated for each sample. Colonies (aggregates containing 40 or more cells) were scored at day 7 and day 14 in marrow cultures and at day 10 in peripheral blood cultures. Early (type 1) CFU-GM give rise to colonies after 10–14 days of incubation and represent a more immature precursor of the granulocytes and monocytes than the late (type 2) CFU-GM, which form colonies in 7 days of culture.25

RESULTS

Table 1 lists the monoclonal antibodies directed against leukemic cells that were utilized for these studies. The surface antigens recognized by these antibodies were identified in several cases by immunoprecipitation.

Table 2 lists the percent reactivity of normal hematopoietic cells with the monoclonal antibodies after fluorescence-activated sorting of positive and negative populations. The composition of each population was determined by morphological examination of cytocentrifuge preparations as described previously.2-16-20

Table 3 lists the reactivity of normal myelomonocytic colony-forming progenitors with the 15 antibodies. Antibodies R1B19, S4-7, S3-13, S8-6, S16-109, S16-144, S1-19-9, and S17-12 were tested using complement-dependent cytotoxicity. R1B19, S4-7, and S3-13 were also tested in cell sorting experiments, and the other 7 noncytotoxic antibodies were tested by cell sorting only. Antibodies S4-7 and R1B19 recognized all late CFU-GM, but were partially or completely nonreactive, respectively, with early CFU-GM. Antibodies S3-13, S8-6, S16-144, S16-109, S1-19-9, and LB3-45 recognized virtually all colony-forming cells.
Table 1. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Immunogen</th>
<th>Immune Response</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1B19</td>
<td>IgM</td>
<td>HL-60 (M3)</td>
<td>Secondary</td>
<td>gp145, gp105 (carbohydrate moiety)</td>
</tr>
<tr>
<td>S4-7</td>
<td>IgM</td>
<td>KG1 (M3)</td>
<td>Secondary</td>
<td>gp150 (carbohydrate moiety)</td>
</tr>
<tr>
<td>S1-37</td>
<td>IgG2a</td>
<td>AMV-P†</td>
<td>Secondary</td>
<td>gp43</td>
</tr>
<tr>
<td>LB3-37</td>
<td>IgG2a</td>
<td>AML (M1)</td>
<td>Secondary</td>
<td>ND</td>
</tr>
<tr>
<td>S17-12</td>
<td>IgG2b</td>
<td>BV173 (CML)‡</td>
<td>Primary</td>
<td>p23</td>
</tr>
<tr>
<td>S16-109</td>
<td>IgG2b</td>
<td>K562 (CML)</td>
<td>Secondary</td>
<td>ND</td>
</tr>
<tr>
<td>S16-144</td>
<td>IgG2b</td>
<td>K562 (CML)</td>
<td>Secondary</td>
<td>ND</td>
</tr>
<tr>
<td>LB3-45</td>
<td>IgG2a</td>
<td>AML (M1)</td>
<td>Secondary</td>
<td>ND</td>
</tr>
<tr>
<td>S6-8</td>
<td>IgM</td>
<td>AML (M4)</td>
<td>Primary</td>
<td>p67</td>
</tr>
<tr>
<td>S3-13</td>
<td>IgM</td>
<td>AML (M1)</td>
<td>Primary</td>
<td>p29</td>
</tr>
<tr>
<td>S5-7</td>
<td>IgG1</td>
<td>ML3 (M3)</td>
<td>Secondary</td>
<td>p20</td>
</tr>
<tr>
<td>S17-14</td>
<td>IgG3</td>
<td>BV173 (CML)</td>
<td>Primary</td>
<td>ND</td>
</tr>
<tr>
<td>S16-89</td>
<td>IgG2a</td>
<td>K562 (CML)</td>
<td>Secondary</td>
<td>ND</td>
</tr>
<tr>
<td>L5-1</td>
<td>IgG2a</td>
<td>HL-60 (M3)</td>
<td>Secondary</td>
<td>gp90 (transferrin receptor)</td>
</tr>
<tr>
<td>SK37-7</td>
<td>IgG1</td>
<td>Melanoma</td>
<td>Secondary</td>
<td>gp28, gp33 (HLA-DR)</td>
</tr>
<tr>
<td>S1-19-9</td>
<td>IgG2b</td>
<td>WT-52†</td>
<td>Primary</td>
<td>gp28, gp33 (HLA-DR)</td>
</tr>
</tbody>
</table>

*AMV-transformed cells.*
†EBV-transformed B lymphocytes.
‡Chronic myelogenous leukemia.

These results have already been partially published.
†Percent positive cells by indirect immunofluorescence. Results are from at least three experiments with different marrow samples.
‡Including 75%-90% of proerythroblasts and basophilic erythroblasts.
§Very low fluorescence intensity.

whereas antibodies S17-12, LB3-37, S1-37 showed virtually no reactivity with these progenitors.

Reactivity With Acute Myelogeneous Leukemias

Figure 1 shows the reactivity of 63 cases of adult and childhood AML with the monoclonal antibodies described. The percentage of cells reactive with each antibody varied among donors: for each antibody, some cases were strongly reactive and other cases completely nonreactive. The composite phenotype of each case of leukemia was unique to each patient and did not correlate with the classes of the FAB classification (Fig 2, A–E). There was no detectable difference in reactivity between pediatric and adult cases (not shown). Furthermore, in almost no cases did the individual markers tested react with more than 60%-70% of the cells analyzed by cytofluorimetry.

The heterogeneity of antigen expression among

Table 2. Reactivity of Normal Hemopoietic Cells With Monoclonal Antibodies as Determined by Cell Sorting*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Erythroblast</th>
<th>Myeloblast</th>
<th>Promyelocyte</th>
<th>Myelocyte</th>
<th>Metamyelocyte</th>
<th>Granulocyte</th>
<th>Monocyte</th>
<th>T Lymphocyte</th>
<th>Non-T Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1B19</td>
<td>0†</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S4.7</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>50–70</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S1.37</td>
<td>0</td>
<td>0</td>
<td>30–50</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LB3.37</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>70–90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S17.12</td>
<td>10–20‡</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S16.109</td>
<td>&gt;10–15‡</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>50–75</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S16.144</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>50–60</td>
<td>50–60</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LB3.45</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>50–70§</td>
<td>0–20§</td>
<td>&gt;90</td>
<td>0</td>
</tr>
<tr>
<td>S8.6</td>
<td>0</td>
<td>&gt;90</td>
<td>40–50</td>
<td>0</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>60–70</td>
</tr>
<tr>
<td>S3.13</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20–40</td>
<td>30–50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S5.7</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>0</td>
<td>60–70</td>
<td>&gt;90</td>
<td>0</td>
<td>&gt;90</td>
</tr>
<tr>
<td>S17.14</td>
<td>0</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>0</td>
<td>70–85</td>
<td>70–85</td>
<td>50–70</td>
<td>0</td>
</tr>
<tr>
<td>S16.89</td>
<td>&gt;90</td>
<td>10–20</td>
<td>20–30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L5.1</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>30–40</td>
<td>0</td>
</tr>
<tr>
<td>SK37.7</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>30–40</td>
<td>0</td>
</tr>
<tr>
<td>S1.19-9</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;90</td>
<td>0</td>
<td>30–40</td>
<td>0</td>
</tr>
</tbody>
</table>

*These results have already been partially published.
†Percent positive cells by indirect immunofluorescence. Results are from at least three experiments with different marrow samples.
‡Including 75%-90% of proerythroblasts and basophilic erythroblasts.
§Very low fluorescence intensity.
cases of AML and among subpopulations within a given patient with AML is illustrated in Table 4 and Fig 3. Cells from five AML patients were double-labeled with biotinylated, Avidin Texas Red-tagged monoclonal antibody and fluoresceinated antibody in three separate experiments. No unique or predictable pattern of expression was seen in the three cases of M4 AML. Furthermore, varying percentages of cells from each of the five patients expressed one, both, or neither of the antigens detected by the antibodies. Variations as great as 20% in reactivity of single antibodies with cells of the same patient were observed in different experiments using different combinations of antibodies. This different reactivity may reflect either different sensitivity of the two fluorochromes or variable interactions between the two combinations of antibodies, so that the presence of antibody to one antigen interferes with the detection of the second.

**DISCUSSION**

AML is believed to result from impairment in the well regulated processes of maturation and differentiation. This impairment is manifested as follows: proliferation without maturation; expression of antigens present normally only in small amounts in fetal or immature cells; and coexpression of two or more mutually exclusive lineage-related antigens. Because of the great numbers of immature myelomonocytic precursors, the leukemic marrow in AML appears relatively homogeneous in comparison to the normal marrow. This apparent homogeneity has been questioned recently, and much current work has focused on the actual heterogeneity in the leukemic population.

The experiments described in this report were performed to examine more closely the heterogeneity in AML. We have generated and characterized a panel of 15 monoclonal antibodies that react with lineage-related and stage-related antigens expressed on normal myelomonocytic cells. Cells from 63 patients with AML were tested with the panel of antibodies using indirect immunofluorescence, cell-sorting, complement-mediated cytotoxicity, and double-labeling. The immunofluorescence and cytotoxicity studies showed that the surface phenotype of the leukemic population in any single patient was comprised of numerous subpopulations that expressed none, some, or all of the antigens present on normal hemopoietic precursors. The particular combinations of antigens were not characteristic of any single normal cell type recognizable by morphological or clonogenic analysis. Furthermore, the composite antigenic phenotype among the 63 patients did not correlate with any morphologically recognizable subtypes of AML as determined by the FAB classification. Finally, many cells within the leukemic population failed to express any myelomonocytic determinants, such as R1B19 or S4-7, or any markers seen in normal CFU-GM, such as S8-6, S16-144, or S3-13.

These data can be interpreted in several ways. Conceivably, the leukemic transforming event occurs in a cell less mature than the early CFU-GM, and the cells never develop lineage-related or stage-related antigens. This notion finds support in the work of Fialkow who showed that, in some cases of AML, in
HETEROGENEITY IN ACUTE MYELOID LEUKEMIA

**Fig 2.** (A–E) Reactivity of monoclonal antibodies to leukemic cells classified according to FAB morphological subtypes M1–M5. Reactivity was determined as described in the legend to Fig 1.

Females heterozygous for glucose-6-phosphate dehydrogenase (G6PD), the leukemic monoclonal G6PD isoenzyme pattern was present in both erythroid and myeloid precursors.\(^{28}\) Likewise, the coexpression of lymphoid and myeloid or myeloid and erythroid antigens in some leukemia cells suggests that the leukemia arose in a multipotent hemopoietic cell.\(^{29}\)

Alternatively, it is possible that the heterogeneity of the leukemic population simply reflects lack of synchronization: anti-DR, for example, appears to be S
phase-related and, obviously, not all leukemic cells are in S phase. Other antigen expression may also be related to the cell cycle or to the metabolic status of the individual cell. In this case, leukemic subpopulation heterogeneity might be trivial biologically, but important clinically if monoclonal antibodies are used for diagnosis and treatment.

Furthermore, the subpopulation heterogeneity may represent the culmination of aborted differentiation of the different leukemic subpopulations. This interpretation would seem to support the model recently proposed by McCulloch, which postulates a highly disorganized gene expression at the origin of leukemia, rather than a block of differentiation or an uncoupling between self-renewal and differentiation. The high degree of heterogeneity of cell surface phenotypes, even within the same leukemia population, seems to suggest an abnormal gene expression with evidence for both interlineage and intralineage infidelity. The marked antigenic heterogeneity of AML cells seems to prevent the possibility of subclassification of AML into antigenically distinct subgroups with possible different prognosis, as demonstrated for ALL. Recently, a partial correlation between antigenic phenotype and cytometry of AML has been reported using small panels of monoclonal antibodies. 12, 32 On the other hand, marked antigenic heterogeneity, without correspondence to normal phenotypes, has been demonstrated among lymphoblasts of T-ALL, 33 usually considered as malignant clones of cells at defined stages of T cell differentiation. 14 These discrepancies probably derive from the different monoclonal antibodies used (larger panels can probably detect more antigenic heterogeneity in both AML and ALL) and from the different percentages of cells positive in immunofluorescence arbitrarily considered as a limit for considering a leukemia as reactive to a monoclonal antibody.

The highly variable antigenic expression of AML cells probably represents a relevant problem for the therapeutic use of monoclonal antibodies. Our results show that the reactivity of a monoclonal antibody to more than 90% of AML cells is a rare event. However, most of AML blasts are known to represent cells with a low or absent proliferative ability. 26 Studies on AML clonogenic cells in vitro could bring about information on the antigenic phenotype of the AML cells with high proliferative potential. Hence it should be possible to determine if biologic basis exists for a therapeutic use of monoclonal antibodies in these leukemias.

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