A New Acute Leukemia-Associated Blast Cell Antigen Detected by a Monoclonal Antibody

By Ronald Billing, Kathy Lucero, Bing J. Shi, and Paul I. Terasaki

A monoclonal mouse antibody has been raised to the common acute lymphoblastic leukemia (cALL) cell line Reh. It is a cytotoxic antibody of the IgG2a subclass that reacts with leukemia cells from the following patients: 69% non-B non-T ALL, 50% T-ALL, 18% acute myeloblastic leukemia (AML), and 66% chronic myeloid leukemia (CML) blast crisis lymphoid cells. Other types of leukemia and all normal blood cells tested were negative, including T and B lymphocytes, granulocytes, monocytes, erythrocytes, and spleen cells. The detected antigen appears to be a type of blast cell antigen because it is also present on phytohemagglutinin (PHA) blast cells, myeloblast from normal bone marrow cells (by CFU-C), and all lymphoblastoid cell lines tested. Only one active antibody species could be detected by preparative isoelectric focusing on polyacrylamide gels and by protein-A-Sepharose affinity chromatography.

HETEROANTIBODIES produced mainly in the rabbit and directed against leukemia-associated cell surface antigens have increased our knowledge of the origins and nature of the different forms of leukemia. Some of these reagents have prepared the way for more accurate diagnosis of leukemia and new possibilities for immunotherapy. For example, Ia and T-cell antisera can be used to differentiate, non-T non-B ALL from T-ALL, and antisera against the cALL antigen (p98) can recognize cALL and lymphoid-type CML blast crisis. Some antisera have been used as an early warning of relapse by immunologically detecting recurrent leukemia cells in the bone marrow. An innovative approach to the use of antisera in immunotherapy has been the use of anti-cALL sera to remove leukemia cells from autologous bone marrow in vitro prior to transplantation.

These approaches have been given an added impetus by the recent development of the hybridoma technology to produce monoclonal antibodies. Hybridoma-produced antibodies offer several advantages over previously produced heteroantisera antibodies. First they are readily available and high titered; second, there is no need to absorb out unwanted antibody specificities, which was almost always a problem with heteroantisera; and third, illustrated by our present findings, a wider variety of antigenic determinants may be recognized by the hybridoma technique, with the prospect of finding previously undetected antigens.

MATERIALS AND METHODS

Cells

Heparinized peripheral blood samples were drawn from children and adults with a diagnosis of acute leukemia. The ALL and AML patients had peripheral blood blast counts of 90% or greater. Leukemia cells from patients and peripheral blood lymphocytes from healthy donors were isolated by Ficoll-Hypaque density gradient centrifugation. Leukemia cells were stored in liquid nitrogen.

Cell lines were grown in suspension cultures in RPMI 1640 containing 20% heat-inactivated fetal calf serum.

B-cell-enriched and T-cell-enriched lymphocytes were prepared from unfractionated lymphocytes by rosette formation of the T cells with neuraminidase-treated sheep red blood cells (SRBC). The rosetted lymphocytes were centrifuged through Ficoll-Hypaque. The T cells sedimented to the bottom of the tube and the B cells remained at the interface. The T cells were recovered by lysis of the SRBC with hypotonic Hanks' balanced salt solution. The purity of the fractionated B cells measured by cytotoxicity tests with rabbit anti-B cell antisera was in the range of 80%–100%, whereas T-cell preparations showed less than 10% B-cell contamination by the same method. Ninety-five percent or greater of the T cells formed E rosettes.

Cytotoxicity

A microcytotoxicity test was performed by addition of 1 μl of antiserum at various dilutions to 1 μl of cell suspension (2000 cells) in a microtest plate. The mixture was incubated at 22°C for 30 min, 5 μl of rabbit complement was added, and incubation was continued for an additional 60 min. The reaction was then stained with eosin dye, fixed, and read for cytotoxicity.

Production of Monoclonal Antibodies

Two 6-wk-old female BALB/c mice were immunized intravenously with 1–2 x 10⁷ Reh cells (cultured cALL cell line) weekly for 3 wk. Three days following the final injection the spleens were removed and a single cell suspension was made. Spleen cells were fused with P3X63-Ag8 myeloma cells at a ratio of 7:1 with 50% polyethylene glycol. After the fusion, hybrid cells were selected by hypoxanthine/aminopterin/thymidine (HAT) treatment. After 3 wk, supernatants were screened by microcytotoxicity against Reh cells. Several wells produced discriminating antibodies. One hybridoma, B5, which showed specificity for ALL cells, was cloned and grown out in tissue culture flasks and as ascites in BALB/c mice.

Purification of Monoclonal Antibody by Protein-A-Sepharose

Sixty milliliters of B5 culture supernatant was applied to a protein-A-Sepharose affinity column and eluted with different pH
buffers according to Ey, Prowse, and Jenkins. Phosphate and acetate buffers were used instead of citrate to avoid the chelating effect which would inhibit the complement-dependent cytotoxicity reaction.

Complement-Dependent Cytotoxicity Against Stem Cells

Complement-dependent activity against the committed myeloid stem cells designated as colony-forming unit culture (CFU-C) were assayed by a modification of the cytotoxicity test described above. One-tenth milliliter of the test antibody was added to 0.1 ml of bone marrow cells (10⁶ cells/0.1 ml) and the combination was mixed and incubated at room temperature for 30 min. We then added 0.1 ml of fresh-frozen (-70°C) normal rabbit serum to the mixture and continued incubation for 60 min. After incubation with antibody and complement, bone marrow cells at 2 x 10⁷/ml in 0.3% agar were plated over white cell feeder layers, incubated at 37°C in an atmosphere of 7.5% CO₂ in air for 10 days, and the number of granulocyte-macrophage colonies containing greater than 40 cells was enumerated under a dissecting microscope.

RESULTS

Both B5 ascites fluid and culture supernatant were tested at serial dilutions against a variety of normal blood cells and leukemia cells from a large number of donors. There was no detectable difference in specificity between the ascites and the culture supernatant; however, the ascites fluid titer was 1000-fold higher than that of the supernatant. The cytotoxic titer of the ascites fluid against the immunizing cell and peripheral blood ALL cells was 1:10⁴ whereas at a dilution of 1:10 there was no detectable cytotoxicity against normal peripheral blood B and T lymphocytes (Fig. 1).

B5 ascites fluid showed less than 10% killing at a dilution range of 1:10–1:10⁶ against a panel of various normal blood cells, including T and B lymphocytes, spleen cells, granulocytes, and monocytes (Table 1). However, B5 ascites were strongly positive against all 10 culture cell lines tested. The lines tested originated from various sources (Table 1): T-ALL, cALL, AML, CML, lymphoma, and Epstein-Barr virus transformed normal B lymphocytes.

B5 was positive against more cases of ALL than any other subclass of leukemia (Table 2). Sixty-nine percent of non-B, non-T ALL and 50% of T-ALL were positive. The lymphoid type of CML blast crisis was also highly positive (66%), whereas other types of CML were negative. Apart from 9/50 AML cases, all other leukemia cells were negative.

The B5 antibody was tested by indirect immunofluorescence using goat anti-mouse IgG FITC and goat anti-mouse IgM FITC as a second antibody. Positive fluorescence was only observed with anti-mouse IgG, and a 3-layer system showed the positive antibody was IgG₃. Although fewer cells were tested by immunofluorescence, the specificity did not appear to be different from that obtained by cytotoxicity. ALL cells, lymphoblastoid cell lines, and PHA blasts were all positive, whereas normal lymphocytes were negative.

When normal bone marrow was treated with B5 ascites and complement, no myeloid colonies (CFU-C) could be grown out in soft culture (Table 3). Therefore, the B5 antibody reacted with an antigen either present on the myeloid stem cell itself or on an accessory cell essential for its growth. Less than 5% of normal bone marrow cells were positive by immunofluorescence.

By Ouchterlony immunodiffusion, the B5 supernatant contained two mouse immunoglobulins (Ig), IgG₁ and IgG₂a. These Ig subclasses were separated by affinity chromatography on protein-A bound to Sepharose 4B (Fig. 2). Different mouse Ig have different binding affinities to protein-A and can be eluted by lowering the pH of the elution buffer. Peak 1 (the unbound fraction) contained mainly fetal calf proteins and small molecules, such as amino acids, which were present in the growth medium. Peaks 2 and 3, which were IgG₁, were noncytotoxic and probably repre-

![Graph showing Cytotoxicity against ALL (O-O) and normal B or T cells.](image)

Table 1. Cytotoxicity of Monoclonal Antibody B5 From Ascites Against Various Normal White Blood Cells and Lymphoblastoid Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>No. Positive/No. Donors Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal T lymphocytes</td>
<td>0/20</td>
</tr>
<tr>
<td>Normal B lymphocytes</td>
<td>0/5</td>
</tr>
<tr>
<td>Normal spleen</td>
<td>0/20</td>
</tr>
<tr>
<td>Normal granulocytes</td>
<td>0/11</td>
</tr>
<tr>
<td>PHA blasts</td>
<td>13/13</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0/9</td>
</tr>
<tr>
<td>Cell lines†</td>
<td>10/10</td>
</tr>
</tbody>
</table>

* A negative reaction was less than 10% killing at a dilution of 1:10. A reaction was considered positive if more than 60% of the cells were killed at a dilution to 1:10⁶.

†Reh (cALL); Daudi, Raj (Burkitt's lymphoma); Mol, 4, 8402, HSB2 (T-ALL); K562 (CML); Cole, HL60 (AML) IM (normal B line).
sent IgG1 from the genetic information present in the parental myeloma line, X63, which is known to secrete IgG1. Peak 4, which was found to be IgG2 by immunodiffusion, contained the only cytotoxic fractions. No protein was eluted with glycine buffer, pH 3.0.

The monoclonal characteristics of the B5 antibody was demonstrated by preparative isoelectric focusing (Fig. 3). One microliter of B5 ascites was focused on a 5.5% polyacrylamide disc gel containing ampholytes with a pH range of 6.0–8.0. After focusing, the gel was sliced into 50 2-mm slices. Each slice was eluted with 200 μl of 0.1 M phosphate buffer 0.15 M NaCl, which was tested by cytotoxicity for antibody activity. Most of the cytotoxic activity appeared in fraction 9, which had a pH of 7.65.

**DISCUSSION**

The B5 monoclonal antibody described here appears to be directed against a previously unreported leukemia-associated antigen. This antigen is not expressed by any normal peripheral blood cells or cells from patients with CLL, hairy cell leukemia, or Sézary cell leukemia. It was present on leukemia cells from nearly 70% of patients with non-B non-T ALL and some patients with T-ALL, AML, and CML. The antigen is also present on some normal blast cells, such as PHA blasts of normal T lymphocytes. Growth of myeloid stem cells in soft agar (by CFU-C) was eliminated by treatment of the bone marrow cells with B5 and complement. Therefore, the B5 antigen is present either on myeloid stem cells or on an accessory stem cell needed for the growth of myeloid colonies.

By protein-A chromatography, immunodiffusion, and indirect immunofluorescence using antibodies directed against mouse immunoglobulin subclass, the B5 antibody appears to be of the IgG2a subclass. By preparative isoelectric focusing only one peak of activity was found, indicating that the active antibody

**Table 2.** Cytotoxicity of Monoclonal Antibody B5 From Ascites Fluid Against Leukemia Cells From Various Patients

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No. Positive*/No. Patients Tested</th>
<th>Percent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-B, non-T ALL</td>
<td>29/42</td>
<td>69%</td>
</tr>
<tr>
<td>T-ALL</td>
<td>4/8</td>
<td>50%</td>
</tr>
<tr>
<td>AML</td>
<td>9/50</td>
<td>18%</td>
</tr>
<tr>
<td>AMML</td>
<td>0/4</td>
<td>0%</td>
</tr>
<tr>
<td>CML chronic</td>
<td>0/3</td>
<td>0%</td>
</tr>
<tr>
<td>CML lymphocytic BC</td>
<td>4/6</td>
<td>66%</td>
</tr>
<tr>
<td>CML myelocytic BC</td>
<td>0/1</td>
<td>0%</td>
</tr>
<tr>
<td>CLL</td>
<td>0/12</td>
<td>0%</td>
</tr>
<tr>
<td>Sézary</td>
<td>0/3</td>
<td>0%</td>
</tr>
<tr>
<td>Hairy cell</td>
<td>0/2</td>
<td>0%</td>
</tr>
</tbody>
</table>

*A negative reaction was less than 10% killing at a dilution of 1:10. The positive reactions had more than 80% killing at all dilutions to 1:10°.*

†BC, blast crisis.

**Table 3.** Effect of B5 Monoclonal Antibody on Human CFU-C

<table>
<thead>
<tr>
<th>Pretreatment of Bone Marrow Cells</th>
<th>Titer of B5</th>
<th>C′</th>
<th>Colonies/2 x 10³ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:100</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1:10,000</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1:100,000</td>
<td>+</td>
<td>99</td>
</tr>
</tbody>
</table>

*C′, rabbit complement treated.*

Fig. 2. B5 supernatant fractionated by protein-A-Sepharose CL-4B column chromatography. (◊◊◊) OD 280nm.

Fig. 3. Preparative isoelectric focusing of B5 on 5.5% polyacrylamide gels. (◊◊◊) Cytotoxicity of each fraction.
appears to be monoclonal. The IgG1 antibody, which was secreted by the X63 myeloma 8, was not reactive. Other monoclonal antibodies produced in our laboratory but having different specificities have had clearly different isoelectric points.

Serologic data obtained with the B5 antibody indicate that it is not directed against the cALL antigen.23 The cALL antigen is found on 70% of non-B non-T ALL cells, rarely on AML cells, and is not present on CFU-C or PHA-stimulated lymphocytes. Of the lymphoblastoid cell lines tested in this study, only Reh has the cALL antigen. The B5 clearly reacts with more than cALL antigen positive cells, being cytotoxic to cells from 20% of the patients with AML, all of the cell lines tested, and PHA blasts. Although it reacted with nearly 70% of cells from patients with non-B non-T ALL, some of these did not express the cALL antigen. The breakdown of the subclasses of non-B non-T ALL was as follows: B5 + cALL + 21 cases, B5 + cALL – 6, B5 – cALL + 8, B5 – cALL – 7.

Judd et al.12 have described a blast antigen with a molecular weight of 90,000 under reducing conditions and 180,000 under nonreducing conditions. Three monoclonal antibodies 5E9, B3/25, and OKT9 have now been described that react with the same antigen.13 15 The antigen has recently been reported to be a cell surface receptor for transferrin.16 We have also previously described a heteroantisera 157 raised to CEM that reacts with a similar antigen, gp 90 dimer (Billing et al.17 and unpublished results). We have extensively compared the reactivity of the heteroantisera 157 with B5 and found them to be different; 157 reacts with monocytes and many AML cells that do not react with B5. Extensive immunoprecipitation experiments have yielded no antigenic peaks with B5. Therefore, B5 does not appear to be reacting against the gp 90. B5 also appears to be different from the T10 monoclonal antibody, which is present on thymocytes (negative to B5) and PHA-stimulated lymphoblasts and recognizes a glycoprotein of 45,000 daltons.15

Molecular weight determination of the B5 antigen has proved to be difficult. Immunoprecipitation studies with B5 and 125I surface membrane labeled antigens have revealed no prominent bands or peaks of radioactivity on SDS gels ranging from 5% to 12% acrylamide. Similar studies with periodate 3H-sodium borohydride labeled carbohydrate membrane antigens have also revealed no radiolabeled peaks. Currently we are investigating other labeling methods and gel filtration to determine the apparent molecular weight of the B5 antigen. It is possible that the B5 antigen is a glycolipid.

Because B5 was reactive with leukemia cells from 29/42 non-T-ALL and 9/50 AML patients, it might be useful in further subclassifying acute leukemia.

ACKNOWLEDGMENT

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REFERENCES