Use of Monoclonal Antibodies to Identify Cerebrospinal Fluid Lymphoblasts in Children With Acute Lymphoblastic Leukemia

By A.C. Homans, E.N. Forman, and B.E. Barker

The identification of small numbers of leukemic cells in the cerebrospinal fluid (CSF) presents a diagnostic problem in the treatment of children with acute lymphoblastic leukemia (ALL). We adapted a latex sphere rosetting technique to allow us to identify simultaneously cell surface markers and cell morphology in 199 CSF samples from 34 patients and 14 control subjects. In patients without leukemic meningitis, the majority of CSF lymphocytes (89%) were found to be mature T cells positive for OKT1. A much smaller number of cells (8%) were found to be B cells positive for Ia. In these children, only 3% of CSF lymphoid cells expressed the common acute lymphoblastic leukemia antigen (CALLA). Similar results were found in the control subjects. By contrast, 28 CSF samples from nine children with varying numbers of CSF lymphoblasts had much greater proportions of CALLA- and Ia-positive CSF cells (24% to 96%). Leukemic meningitis was present in one of these patients and later developed in four others. However, three patients with small numbers of lymphoblasts present but with low proportions of CALLA-positive CSF cells (less than 5%) subsequently had normal CSF examinations. We found the use of this rosetting technique valuable in providing information complementary to that obtained from cell morphology alone about the possible malignant nature of small numbers of lymphoblast-like CSF cells seen on cytocentrifuge preparations in children with ALL.

The prognosis for children with acute lymphoblastic leukemia (ALL) has improved dramatically over the past few decades. Relapse in the central nervous system (CNS) remains a difficult problem, however, occurring in approximately 10% of these patients despite prophylactic CNS treatment consisting of intrathecal medication or cranial radiation or both.1,2 Current ALL treatment regimens monitor cerebrospinal fluid (CSF) samples periodically to check for the development of leukemic meningitis. Overt CNS relapse may be defined as a CSF pleocytosis (greater than 10 mononuclear cells per cubic millimeter) with unmistakably malignant cells present on cytocentrifuged preparations.3,4 However, the examination of CSF samples of many other patients yields suspicious but nondiagnostic results with a CSF mononuclear cell count of less than 10 cells per cubic millimeter and with small numbers of lymphoblast-like and normal lymphocytes admixed on a cytocentrifuged specimen.5,6 In this latter circumstance, the diagnosis of CNS leukemia by morphological criteria alone is confounded, since standard light microscopy has limitations in differentiating small numbers of lymphoblasts from nonmalignant reactive cells of the lymphoid series.5,7

Whereas specific immune phenotyping of lymphoblasts in diagnostic bone marrow specimens has become routine, usual immunologic methods of studying cell surface antigens are inadequate for studying ordinary spinal fluid samples. Conventional immunofluorescent methods require large numbers of cells (0.5 × 10⁶ cells per sample) and thus is only practical either when the CSF cell count is high or when large, technically impractical amounts of spinal fluid are obtained. In these conditions, reports are present in the literature defining T and B lymphocyte populations in isolated patients with neoplastic meningeal disease.8-14 In addition, results of immunofluorescent testing may not be helpful when small numbers of malignant cells are present in mixed mononuclear cell populations. We have adapted a methodology for the identification of specific immune markers for small numbers of CSF cells that, at the same time, preserves excellent cell morphology. Cells in CSF samples from children with ALL who were in CNS remission and from adults without malignancy having myelograms done were studied for the presence of the common ALL antigen (CALLA), OKT1, and the DR-related antigen (Ia). Spinal fluid samples from nine children who had lymphoblasts present in their CSF were also studied. One of the children in this latter group had overt CNS disease as previously defined, and the remainder had only small numbers of lymphoblasts mixed with normal cells. Simultaneous examination of CSF cells for immunologic and morphological characteristics was found to be a valuable addition to standard microscopic examination of spinal fluid cell morphology in clarifying the possible leukemic nature of CSF mononuclear cells.

MATERIALS AND METHODS

Patients and cells. Samples of CSF were obtained from children being treated in the Pediatric Oncology Clinic, Rhode Island Hospital, and from adult control subjects. Most patients were being treated on current Pediatric Oncology Group treatment protocols, and samples were obtained at times specified by those protocols (usually every two months during systemic maintenance therapy). Ordinarily, 3 mL of CSF was obtained from each patient. One milliliter was used for cell count and cytocentrifuge examination for differential, as has been previously described.14 The remaining 2 mL was separated into two aliquots to be used for monoclonal antibody testing. Cells for cell surface antigen testing were obtained by centrifugation of CSF for ten minutes at 800 rpm. The supernatant was sent for routine determinations of protein and glucose. The cell count and cytocentrifuge were performed with no knowledge of the patient's clinical status, and immunosuppressive therapy was studied without knowledge of prior bone marrow lymphoblast antigen testing. Informed consent was obtained for all adult control subjects, and approval of this investigation was given by our institution's Committee on Protection of Human Subjects.

Antibody labeling of CSF cells. A two-stage indirect labeling procedure was followed, similar to the method of Bernard et al.15

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using 10-μL aliquots of a sonicated 1.4% suspension of 1-μm latex spheres (Covasphere MX, Covalent Technology Corp, Ann Arbor, Mich) bound with 10-μL aliquots of goat antimouse immunoglobulin (Tago Corp, Burlingame, Calif) at room temperature. After washing with RPMI 1640 with 10% inactivated fetal calf serum, the immunoglobulin-coated spheres (immunospheres) were stored frozen at −20°C. One-milliliter aliquots of CSF were centrifuged and the cell pellet was incubated at 4°C for 30 minutes in 0.2 mL of RPMI media plus 10% inactivated fetal calf serum with 5 μL of mouse monoclonal antibody. Antibodies used included J5 (Coulter Clone, Hialeah, Fla) to identify CALLA, and OKT11 and OKT1a (Ortho Pharmaceutical, Raritan, NJ) to identify mature T cells and a subset of B cells, respectively. After incubation with the monoclonal antibody, the cells were washed twice with media and subsequently incubated with a 0.2-mL aliquot of the latex suspension. Excess unbound spheres were removed by centrifugation of the sphere-cell mixture through a 1.5-mL layer of fetal calf serum. The cell pellet was incubated at 4°C for 30 minutes in 0.2 mL of RPMI media plus 10% inactivated fetal calf serum with 5 μL of mouse monoclonal antibody. Antibodies used included J5 (Coulter Clone, Hialeah, Fla) to identify CALLA, and OKT11 and OKT1a (Ortho Pharmaceutical, Raritan, NJ) to identify mature T cells and a subset of B cells, respectively. After incubation with the monoclonal antibody, the cells were washed twice with media and subsequently incubated with a 0.2-mL aliquot of the latex suspension. Excess unbound spheres were removed by centrifugation of the sphere-cell mixture through a 1.5-mL layer of fetal calf serum for 12 minutes at 900 rpm. The cell-free supernatant was discarded, and the final mixture of cells and cells bound with spheres was centrifuged on a cytocentrifuge (Cytospin, Shandon Eliott, Sewickley, Pa) at 700 rpm for four minutes. The resulting slide was stained with Wright's stain and examined by light microscopy.

Previous work with blood and bone marrow cells in our laboratory (unpublished observations, 1983) demonstrated that the binding of three or more spheres correlates closely with antigen positivity as measured by visual indirect immunofluorescence. In practice, most positive cells bound many more than three spheres, whereas occasional negative cells may bind one or two immunospheres (cells with a clump of spheres attached to the surface at only one point were not counted as positive). This definition of antigen expression is supported by reports from other researchers using immunosphere methodologies. Monocytes and neutrophils are easily identified with this method because of their ability to phagocytize the spheres. Similar techniques have been used successfully by a number of other investigators to identify specific surface antigens on subsets of peripheral blood and bone marrow cells and found to compare favorably with results obtained by conventional immunofluorescence.

**RESULTS**

Figures 1 and 2 show the ability of this method to identify specific morphological cell types expressing particular surface antigens when they are present in mixed cell populations.

Cell surface antigen determinations were performed on 199 CSF samples from 34 patients and 14 control subjects. One hundred seventy-one CSF samples were obtained from 39 patients to determine the frequency of the cell surface antigens on spinal fluid lymphocytes in patients without leukemic meningitis. One hundred fifty-seven samples were obtained from 25 children with ALL in remission receiving maintenance therapy. These children did not have a CSF pleocytosis nor did they have lymphoblasts or cells suspicious for malignancy seen on morphological examination of CSF cytocentrifuge specimens. Fourteen adult patients having lumbar punctures for myelograms investigating suspected vertebral disk disease served as controls.

As can be seen from Table 1, CALLA-positive CSF lymphocytes were infrequently found in children without CNS leukemia or in adults undergoing myelograms, representing only 3% and 2% of all CSF lymphocytes seen in these patient groups, respectively. Only 8% and 5% of CSF cells were labeled by Ia in these pediatric and adult patients. Cells binding OKT11 on their surface made up the majority of lymphocytes seen, accounting for 69% of CSF lymphocytes in the children and 62% of the CSF lymphocytes in adults.

Cell surface antigens were also investigated in 28 CSF samples from nine children with suspicious or overt leukemic meningitis, of whom seven were studied on sequential lumbar punctures (Table 2). The majority of spinal fluid samples were obtained at times of bone marrow remission, thus eliminating the possibility of contamination of CSF with peripheral blood or bone marrow lymphoblasts. The results presented in Table 2 demonstrate several observations about this methodology. Most of the samples obtained when overt CNS disease was present showed a reasonably good correlation to antigen positivity of initial bone marrow lymphoblasts, when known. In many samples without overt CNS leukemia (samples 7, 9, 11 through 13, 19, 20, 22), the use of immune markers identified more CALLA- or Ia-positive cells than morphologically identified lymphoblasts. These patients subsequently developed overt CNS leukemia. Conversely, patients G, H, and I had samples with single
MONOCLONAL ANTIBODIES FOR CSF LYMPHOBLASTS

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Sample obtained at time of bone marrow relapse.
ND, not done.

Table 1. Cell Surface Antigens on CSF Lymphocytes Obtained by Cytocentrifuge in Patients Without Leukemic Meningitis

<table>
<thead>
<tr>
<th>Monoclonal Antibody Used</th>
<th>No. of Patients</th>
<th>No. of Samples</th>
<th>No. of Mean Lymphs per Sample (Range)</th>
<th>No. of Positive Cells/Total No. of Lymphoid Cells Seen</th>
<th>Percentage Cells Positive (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children with ALL J5</td>
<td>38</td>
<td>84</td>
<td>32 (1-100)</td>
<td>90/2,675</td>
<td>3% (0-10%)</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>5</td>
<td>27 (3-61)</td>
<td>3/133</td>
<td>2% (0-3%)</td>
</tr>
<tr>
<td>Children with ALL Ia</td>
<td>19</td>
<td>23</td>
<td>33 (1-183)</td>
<td>59/760</td>
<td>8% (0-18%)</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>5</td>
<td>42 (2-100)</td>
<td>10/208</td>
<td>5% (0-10%)</td>
</tr>
<tr>
<td>Children with ALL OKT11</td>
<td>30</td>
<td>50</td>
<td>33 (1-155)</td>
<td>1,137/1,644</td>
<td>69% (46-96%)</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>4</td>
<td>23 (5-40)</td>
<td>56/90</td>
<td>62% (53-80%)</td>
</tr>
</tbody>
</table>

Children with ALL were studied while receiving maintenance therapy in bone marrow and CNS remission. J5, anti-common ALL antigen; Ia, anti-HLA Dr.

...lymphoblasts seen but without elevated proportions of CAL-LA-positive cells; these three patients have subsequently had repeatedly normal CSF samples. Patient B died of progressive bone marrow disease without further investigation of CNS malignancy.

In patients D, E, and F, the increased numbers of CAL-LA- and Ia-positive cells in the presence of overt CNS leukemia was accompanied by a marked decrease in the numbers of OKT11-positive cells, indicating that the identification of cells with immunospheres was indeed due to the attachment of spheres to specific cell surface antigens and not to a nonspecific adhesive property of a malignant cell surface.

DISCUSSION

The accurate identification of small numbers of leukemic cells in spinal fluid presents a diagnostic challenge. Simone and others have pointed out the limitations of light microscopy in this situation. Most previous attempts at a more specific immunologic characterization of neoplastic CSF cells have used large volumes of CSF, or samples of CSF with large numbers of cells. In the latter situation, the diagnosis of leukemic meningitis usually is not difficult. A recent report by Casper et al. studied CSF samples of children with ALL with immunofluorescent testing for terminal deoxynucleotidyl transferase (TdT) and demonstrated this to be a viable adjunct to conventional morphological

Table 2. Cell Surface Antigens on CSF Mononuclear Cells in Patients With Visible CSF Lymphoblasts

<table>
<thead>
<tr>
<th>Conventional Cytocentrifuge Data</th>
<th>Monoclonal Antibody Used</th>
<th>No. of Cells Positive/No. of Cells seen</th>
<th>Cells Positive (%)</th>
<th>Initial Bone Marrow Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>J5</td>
<td>7/29</td>
<td>24</td>
<td>59% J5</td>
</tr>
<tr>
<td>B</td>
<td>J5</td>
<td>7/14</td>
<td>50%</td>
<td>52% J5</td>
</tr>
<tr>
<td>C</td>
<td>J5</td>
<td>3/5</td>
<td>60%</td>
<td>40% J5</td>
</tr>
<tr>
<td>D</td>
<td>J5</td>
<td>38/70</td>
<td>54%</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>J5</td>
<td>44/100</td>
<td>44%</td>
<td>ND</td>
</tr>
<tr>
<td>F</td>
<td>J5</td>
<td>6/20</td>
<td>30%</td>
<td>80% J5</td>
</tr>
<tr>
<td>G</td>
<td>J5</td>
<td>1/7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>J5</td>
<td>65/100</td>
<td>65%</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>J5</td>
<td>12/89</td>
<td>13%</td>
<td>90% Ia</td>
</tr>
<tr>
<td>J</td>
<td>J5</td>
<td>0/20</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>J5</td>
<td>91/100</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>J5</td>
<td>3/5</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>J5</td>
<td>1/7</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>J5</td>
<td>91/100</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>J5</td>
<td>5/100</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>J5</td>
<td>2/50</td>
<td>4%</td>
<td></td>
</tr>
</tbody>
</table>

*Sample obtained at time of bone marrow relapse.
ND, not done.
examination of CSF cells. Immunosphere methodology allows simultaneous morphological and immunologic evaluation of the same CSF cell, and any of a wide variety of monoclonal antibodies may be used. CALLA is a convenient antigen to study, since it occurs in approximately 80% of childhood lymphoid leukemias and is found on few normal peripheral blood cells.\textsuperscript{23-25} Our results confirmed this for CSF lymphocytes as well, with only 2% to 3% of spinal fluid lymphocytes from patients without suspected CNS leukemia demonstrating this antigen on their surface with this labeling technique, and with CSF lymphoblasts from children with CALLA-positive leukemias frequently demonstrating this antigen. A somewhat larger number of morphologically normal CSF cells were Ia positive, which is expected, since this antigen may be present on reactive T lymphocytes as well as on some B lymphocytes.\textsuperscript{24,26} Interference by the presence of Ia-positive monocytes is readily discounted with this methodology, since they can readily be seen to be phagocytic for the latex spheres. Although sample numbers were small, we observed the antigen positivity for CSF lymphoblasts to correspond generally with the antigen positivity of diagnostic bone marrow cells. Using this methodology, the majority of normal CSF lymphoid cells were found to be mature T lymphocytes, a finding in agreement with that of most other authors using differing methodologies.\textsuperscript{27-29}

An exception is the letter by Astaldi et al\textsuperscript{8} who noted a marked decrease in the number of CSF T cells identified by sheep erythrocyte rosetting in four patients with ALL.

The immunosphere methodology we have described was found to have several advantages over conventional immunofluorescent techniques for studying spinal fluid cells. It is easily performed in a matter of hours and requires no special fluorescent equipment. In addition, the number of spheres bound to a cell provides easily visible semiquantitative information on the degree of antigen positivity for that cell.\textsuperscript{20} This method offers great flexibility in that the antigens studied may be tailored to the immunophenotype of the neoplastic cells in question. Cell morphology is preserved so that combined morphological and immunologic characterization of specific cells is possible in mixed cell populations and preserved on a permanent slide preparation.

Using this methodology, we studied 28 CSF samples in nine patients with suspected leukemic meningitis and found intriguing results. In two of the patients, immunophenere markers for CALLA and Ia were found on large numbers of cells at times when small numbers of lymphoblasts (less than five) were seen. This finding preceded the development of overt CNS disease in these cases. Of equal clinical importance was the observation in three other patients that when low percentages of CALLA-positive cells (5% or less) were seen in association with occasional CSF blasts, CNS leukemia did not develop.

Another interesting finding was the observation of large numbers of CALLA-positive cells (up to 30) with normal morphology. This is similar to the findings of Casper et al\textsuperscript{7} who, on some occasions, found larger numbers of TdT-positive cells than of lymphoblasts in CSF samples. We have noted the same phenomenon using immunofluorescence for CALLA-positive cells in the diagnostic bone marrow specimens and peripheral blood from two patients with CALLA-positive leukemias. In view of the demonstration of this finding with two separate methods, one might speculate that a larger subset of malignant cells expresses surface antigen changes rather than morphological abnormalities. Alternatively, it may be explained by CALLA eluting from lymphoblasts (a phenomenon observed in vitro with lymphoblast populations\textsuperscript{30} and absorbing onto surrounding normal lymphocytes. This latter explanation would not account for the observation of TdT (a nuclear antigen) in a larger number of cells than in the lymphoblast population.

To our knowledge, this is the first report of methodology using latex immunospheres to identify CALLA and other specific cell surface antigens on CSF cells in children with ALL. This methodology is convenient and provides information that is complementary to the information obtained by examination of cell morphology alone. In several instances, the combined study of immunologic and morphological characteristics was more sensitive than the use of morphological criteria alone in detecting CSF cells that were probably leukemic, in view of the patient's subsequent clinical course. This technique is useful for samples with quite small numbers of cells and may potentially be useful for earlier prediction of CNS leukemia, as was noted in three of our patients. Our results to date using this methodology raise interesting questions about the biology of CNS leukemia.

The observation that morphologically normal lymphoid cells presented antigens found on the malignant cell population needs to be confirmed with larger sample numbers but illustrates the potential limitation of the identification of early CNS leukemia by morphological criteria alone. A multi-institutional, longitudinal study is in progress using this methodology to examine CSF samples in childhood ALL in a larger number of patients to evaluate the prognostic significance of small numbers of CALLA-positive lymphoblasts.

REFERENCES


Use of monoclonal antibodies to identify cerebrospinal fluid lymphoblasts in children with acute lymphoblastic leukemia

AC Homans, EN Forman and BE Barker