The presence of meningeal involvement in children with acute lymphoblastic leukemia (ALL) may have important prognostic and therapeutic implications. Conventional methods of diagnosing central nervous system (CNS) leukemia rely on the interpretation of cerebrospinal fluid (CSF) cell morphology, which may produce ambiguous results in the presence of minimal leukemic involvement. A methodology has been developed for immunophenotyping small numbers of CSF cells while preserving cell morphology. CSF samples from 33 children with CD10 (common ALL antigen [CALLA]) positive ALL were examined at initial presentation using both conventional morphology and this combined immunohistopathologic technique. Six (18%) of the samples contained lymphoblasts or cells considered morphologically suspicious for leukemic involvement. Nine additional samples (27% of the total) had normal CSF morphology, but contained increased numbers of CALLA positive cells. Twelve of the 33 samples were also examined for the simultaneous presence of nuclear terminal deoxynucleotidyl transferase (TdT) and demonstrated increased numbers of cells positive for both TdT and CD10. These data suggest that a large proportion of children with ALL may have abnormalities of CSF cells at initial diagnosis consistent with the presence of occult leukemic involvement.

The improved outcome for acute lymphoblastic leukemia (ALL) in childhood has made it essential to be able to accurately diagnose disease present in pharmacologic sanctuaries at diagnosis and during the course of therapy. In the era before the routine use of therapies specifically targeting leukemia in the central nervous system (CNS), a high proportion of children with ALL relapsed in this site. In addition, children manifesting CNS leukemia at initial diagnosis are felt to have an increased risk of relapse requiring the intensification of both systemic and CNS therapy. Despite the importance attributed to the presence of CNS leukemia, there is little consensus as to the precise cerebrospinal fluid (CSF) cytologic abnormalities considered diagnostic for CNS leukemia, and the incidence of CNS leukemia at diagnosis can vary considerably depending on which diagnostic criteria are used.

Several factors contribute to the difficulty in determining the presence of CNS leukemia, including the small numbers of cells present in routine CSF samples and difficulties in interpreting the significance of morphologic abnormalities of small numbers of CSF cells in cytocentrifuge preparations. The demonstration of leukemia-associated cell surface antigens and nuclear TdT can enhance information available from cell morphology in determining whether or not cells are of leukemic origin. The common ALL antigen (CALLA), designated CD10, has proved to be a useful leukemia-associated antigen and is present on the leukemic cells in over 80% of childhood ALL cases while present on only a few normal lymphocytes. Similarly, nuclear terminal deoxynucleotidyl transferase (TdT) is present in the leukemic cells in over 90% of ALL cases. Whereas immunophenotyping techniques exploiting these and other cell markers are routinely used in examining peripheral blood and bone marrow samples to provide information regarding cell lineage, these techniques are rarely used in examining CSF specimens because of technical limitations imposed by the small cell populations present in most CSF samples. However, several recent reports have studied CSF cells for the presence of TdT or cell surface antigens in patients with malignancy and found these studies to be a valuable adjunct to the examination of cell morphology for determining the presence of CNS malignancy.

In previous studies, we have shown that immunospheres can be a valuable label for identifying CSF cell surface antigens while preserving cell morphology. In the present study, this immunophenotyping technique has been expanded to include the simultaneous detection of two surface antigens and, in some cases, nuclear TdT on CSF mononuclear cells, and we have used this technique to study the CSF cells from children with ALL at initial diagnosis. A hypothesis, based on earlier studies, was that some children with ALL at initial presentation may have a morphologically normal CSF mononuclear cell population but with abnormal CSF cell immunophenotypes, suggesting the presence of occult CNS leukemia. The present data support this hypothesis and suggest that a substantial proportion of children with ALL may have immunologic or histochemical evidence of occult CNS leukemia at initial diagnosis.

MATERIALS AND METHODS

Patients. Children with ALL were eligible for inclusion in this study if they met the following criteria: (1) They were newly diagnosed with non-T, non-B ALL with bone marrow lymphoblasts expressing CD10 (CALLA) and nuclear TdT; and (2) no prior chemotherapy or radiotherapy had been administered. Because there is no adequate correction to account for the presence of
contaminating peripheral blood containing lymphoblasts. Sample
containing red blood cell (RBC) (≥1 RBC per 100x high power
field noted on Wright stained cytocentrifuge preparation) were
excluded. To prevent biasing the result in favor of demonstrating
increased CSF abnormalities, samples in which fewer than 30
lymphocytes were evaluated were eliminated from the data analysis.
This was done so that the presence of a single CD10 positive cell
would not characterize a sample as exceeding the upper limit of
normal range (found to be 3% in our pilot studies). Control patients
included children with leukemia in remission receiving maintenance
therapy (including periodic intrathecal injections), children with
ALL no longer receiving therapy, and children undergoing lumbar
puncture for investigation of possible aseptic meningitis. This study
was approved by the committees for the protection of human
subjects at the participating institutions, and written informed
consent was obtained from control subjects for whom more than
routine amounts of CSF were obtained.

Cell labeling. Cells for immunophenotyping were obtained from
a 1- to 2-mL aliquot of CSF collected during the initial diagnostic
lumbar puncture. Samples from institutions in the New England
Pediatric Oncology Consortium (NEPOC) had media added before
shipping to a central laboratory. All samples were studied within 24
hours of collection. We have previously demonstrated that handling
samples in this manner does not affect surface antigen or nuclear
TdT determinations (unpublished data, 1988). The cell labeling
procedure was performed in a single siliconized glass tube to
minimize possible cell loss during cell washing steps.

The preparation of the antibody-labeled fluorescent microspheres
used a modification of a previously described technique. Monoclonal
antibodies (MoAbs) against cell surface antigens included
anti-CD2 (OKT 11; Ortho Pharmaceutical, Raritan, NJ) and
anti-CD10 (J5; Coulter Clone, Hialeah, FL). The use of CD2
determined an internal control for the nonspecific binding of spheres by
cells since CD2 positive cells would not be expected to be also
positive for CD10. MoAb was incubated with colored fluorescent
copolymer microspheres (Covasphere MX; Duke Scientific, Burlingame, CA). Two dissimilar colors were used: green for CD2 and red for
CD10.

CSF cells were pelleted and resuspended in RPMI 1640 contain-
ing human AB or mouse serum (Dako Corp, Santa Barbara, CA) to
decrease nonspecific antibody binding. The cell pellet was then
incubated with an aliquot of both colors of labeled microspheres.

After incubation, the cells were resuspended and the excess spheres
removed by centrifugation of the suspension through fetal calf serum
(FCS). The remaining cell pellet was cytocentrifuged (Cytospin
FCS) onto glass slides. The slides were prepared from normal peripheral blood mononuclear
cells and known TdT positive lymphoblasts, respectively. Control
slides were fixed and stored with the CSF samples to provide
controls for the presence of TdT and for possible fixation or storage artifact.

Statistics. Percentages of CALLA and TdT positive cells from
the diagnostic and control groups were compared using an unpaired
Student's t-test for means with a two-tailed analysis. Differences
between the proportion of abnormal and normal samples in the
diagnostic and control populations were compared by chi square
analysis.

RESULTS

A total of 61 patients had CSF samples studied at initial
diagnosis. Of these, 28 were excluded due to either RBC
contamination (18 samples) and/or insufficient numbers of
evaluable cells (10 samples) leaving 33 patients available for
comparison with the controls. The clinical characteristics of
these patients demonstrated a median age of 3.75 years
(range, 1.5 to 16 yrs), a median peripheral blood white blood
cell (WBC) of 8,700/μL (range, 1,700 to 176,000). Seven
(21%) of the cases had pre-B leukemia as evidenced by the
presence of cytoplasmic μ chains, and the remainder had
non-T, non-B, non-pre-B ("common") ALL. One hundred
four samples meeting eligibility criteria were studied from
control patients. Of these, 77 were obtained from patients
during maintenance therapy, 19 were from children off
therapy, and 8 were from noneleukemic patients undergoing
investigation for possible meningitis. With a median follow-
up of 25 months, none of the patients has experienced an
isolated CNS relapse.

Findings in the patients studied at diagnosis can be
Grouped according to morphologic, surface antigen, and
cytochemical abnormalities. The median number of CSF
WBC/μL was 0 (range, 0 to 22) with a median of 72
lymphocytes per sample were counted on the immunosphere
preparations. These cell numbers fall in the expected ranges of
CSF counts indicating little cell loss due to immunosphere
typing. Of the 33 samples, 6 (18%) had cells with
morphic abnormalities which were either diagnostic (3
samples) or suspicious (3 samples) for malignancy. The

From www.bloodjournal.org by guest on October 30, 2017. For personal use only.
remaining 27 samples had no identifiable morphologic abnormalities.

Results from immunophenotyping CSF cells (Table 1) show that the majority (73%) of CSF lymphocytes in the control samples were positive for CD2. This proportion of CSF T lymphocytes is in agreement with previously reported ranges of 72% to 93% T cells detected with other methods.\(^{31}\) CD10 positive cells were rarely detected (representing a mean of only 0.8% of the lymphocytes per sample). These results contrast with those in the group of newly diagnosed leukemic patients. The CSF samples obtained from patients at diagnosis showed a small but significant decrease in CD2 positive cells (62% v 72%, \(P = .05\) in controls) with an increase in CD10 positive cells. As expected, the percentage of CALLA positive cells was markedly increased in the CSF cells at diagnosis. We defined an "abnormal" specimen as one containing CD10 positive cells greater than 2 standard deviations above normal (ie, >3% CD10 positive cells per sample). Using this criteria, 9 of 27 children with ALL at diagnosis showed an increased sensitivity for the detection of CNS leukemia with these techniques. The immunosphere label- ing technique circumvents some of the methodologic difficulties in CSF analysis by simultaneously identifying multiple cell characteristics including cell morphology on individual CSF cells in a single aliquot of CSF. Unlike some previous

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>ALL at Diagnosis ((n = 27))</th>
<th>Control ((n = 104))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 10 positive (%)</td>
<td>5.4 ± 11</td>
<td>0.8 ± 1.1</td>
<td>(P = 0.001)</td>
</tr>
<tr>
<td>CD 2 positive (%)</td>
<td>62.0 ± 22</td>
<td>73.0 ± 16</td>
<td>.02</td>
</tr>
<tr>
<td>TdT positive (%)</td>
<td>7.2 ± 17</td>
<td>0.8 ± 1.0</td>
<td>.05</td>
</tr>
<tr>
<td>TdT and CD 10 positive (%)</td>
<td>(\text{simultaneous})</td>
<td>6.0 ± 16</td>
<td>.05 (v 0.19)</td>
</tr>
</tbody>
</table>

Results of CSF lymphocyte immunophenotyping expressed as percent of lymphocytes positive per sample (± SD). Groups tested for TdT are a subset of the groups tested for CD 10 and CD 2.

DISCUSSION

As early improvements in the control of systemic leukemia occurred, the importance of CNS leukemia became apparent. In studies performed before the institution of routine CNS therapy, 40% to 60% of the patients developed evidence of leukemic infiltration of the meninges.\(^{1,3}\) This high rate of CNS relapse led to a diligent search for CNS disease at diagnosis and the institution of early CNS "prophylaxis" with intrathecal chemotherapy and/or radiation. Unfortunately the detection of initial CNS leukemia may be hampered by the small number of cells present in most CSF samples, the presence of contaminating peripheral blood cells, and the difficult, somewhat subjective, nature of interpreting individual CSF cell morphology on cytospin preparations.\(^{6,9}\) Given these difficulties it is not surprising that there has been a lack of consensus regarding the definition of CNS leukemia, and that the proportion of patients with initial CNS disease ranges from 1% to 15% in various studies depending on the diagnostic criteria used.\(^{9}\) To more easily compare the results of various treatment regimens, standardized criteria have been proposed for the diagnosis of CNS leukemia\(^{13}\); however, these criteria remain somewhat arbitrary. Initial CNS involvement is felt by many investigators to be an adverse prognostic indicator, although its importance may be diminished in studies using more intensive therapy when examined by multivariant analysis.\(^{24}\)

Given the possible significance ascribed to initial CNS leukemic involvement, it may be of importance to be able to accurately identify small numbers of leukemic cells in the CSF. Several studies have demonstrated the utility of examining CSF samples for cytochemical or surface antigen characteristics for detecting early CNS relapse\(^{13,16,35,36}\) and showed an increased sensitivity for the detection of CNS malignancy with these techniques. The immunosphere labeling technique circumvents some of the methodologic difficulties in CSF analysis by simultaneously identifying multiple cell characteristics including cell morphology on individual CSF cells in a single aliquot of CSF.
studies, we found rare TdT positive cells in control CSF samples. This finding may be due to an increased sensitivity of the immunoperoxidase method compared with immunofluorescence. The examination of both TdT and CD10 appears to provide greater specificity since there was one patient each with abnormalities of one, but not both, characteristics. When this methodology was used to study initial CSF samples in children with ALL, 45% of the patients were found to have increased numbers of CALLA and/or TdT positive cells, many in the absence of morphologic abnormalities. A recent study by Hooijkaas et al demonstrated that 26% of 43 patients with ALL or non Hodgkin’s lymphoma had increased TdT positive CSF cells at diagnosis. Given the relatively small sample size, the 95% confidence limits for this percentage (14% to 41%) overlaps considerably with our own data (28% to 64% abnormal with 95% confidence limits) showing the two series to be in agreement.

Several explanations could be formulated to account for the high number of patients demonstrating CSF abnormalities. The CSF samples could contain peripheral blood lymphocytic cells introduced during CSF collection, but this seems unlikely for the reasons previously mentioned. The CD10 positive cells may also represent a false positive result. However, the internal controls (simultaneous enumeration of CD2 for detecting nonspecific adhesion of immunospheres) and the simultaneous expression of nuclear TdT argues against this explanation. Finally, our patient population could have inadvertently represented the selection of a group of patients at high risk for CNS leukemia. However, the patients’ ages, WBC, and immunophenotype do not suggest such a selection. The exclusion of samples with fewer than 30 observed lymphocytes did not bias the study’s conclusions since the inclusion of these samples, in fact, increases the proportion of abnormal samples noted (data not shown). It should be noted that the high incidence of morphologic abnormalities reflects the inclusion of samples with “suspicious” morphology in this group.

The most likely explanation for the findings in this study is that the incidence of occult leukemic infiltration of the CNS in children with ALL at diagnosis is much higher than is appreciated by morphologic criteria. Other investigators have found occult CNS abnormalities in a proportion of children with ALL at diagnosis with a variety of techniques including EEG, myelin basic protein analysis, and computerized tomography. These data along with the results of the present study, suggest that a substantial proportion of all pediatric patients with ALL may have some degree of CNS involvement at initial diagnosis. Unfortunately, a drawback of all cell labeling techniques is that the presence of contaminating peripheral blood cells in a CSF sample limits the test’s utility since lymphoblasts may potentially be introduced at the time of the traumatic lumbar puncture.

While these data are in interest with regards to the pathogenesis of CNS leukemia, their therapeutic significance remains unclear. None of our patients with immunohistologically abnormal CSF at diagnosis has suffered a CNS relapse. This contrasts with the findings in the study by Hooijkaas in which 6 of 11 patients with CSF immunophenotypic abnormalities at diagnosis later relapsed in that site within the same time period of observation as in the current study. This may be explained by the more intensive intrathecal CNS prophylaxis administered to all of the patients in our study. Given the effectiveness of current CNS prophylaxis, several hundred patients would need to be studied for extended periods of time to confidently detect small differences in CNS relapse rates between immunophenotypically normal and abnormal patient groups. Indeed, a recent study by Gilchrist et al involving over 1,500 patients did not demonstrate an increased incidence of CNS relapse in children with ALL with CSF blasts present at diagnosis in the absence of CSF pleocytosis.

The findings of the present study may also have importance in interpreting the results of studies assessing the toxicity ascribed to current CNS prophylaxis regimens, because it could be speculated that occult CNS disease present at diagnosis may contribute to CNS injury. The findings of the present study, if confirmed, strengthen the assertion that current CNS “prophylaxis” is, in fact, presymptomatic therapy, and could potentially be useful identifying groups of patients requiring more or less intensive presymptomatic CNS therapy.

ACKNOWLEDGMENT

We acknowledge the assistance of the data managers from the participating institutions for making this study possible, and the technical assistance of the laboratory personnel at Rhode Island Hospital. In addition we acknowledge the contribution of CSF samples by Dr Susan McIntosh, and from the Children’s Cancer program at the Boston Floating Hospital, and of control CSF samples by Dr William Lewander (RIH).

REFERENCES

9. Lauer S, Kirchner P, Camitta B: Identification of leukemic...


Immunophenotypic characteristics of cerebrospinal fluid cells in children with acute lymphoblastic leukemia at diagnosis

AC Homans, BE Barker, EN Forman, CJ Jr Cornell, JD Dickerman and JT Truman