

Immunophenotypic Characteristics of Cerebrospinal Fluid Cells in Children With Acute Lymphoblastic Leukemia at Diagnosis

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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 con-

tained 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.

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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.¹⁻³ 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.^{4,5} 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,⁶⁻⁹ and the incidence of CNS leukemia at diagnosis can vary considerably depending on which diagnostic criteria are used.⁸

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.^{7,9,10} 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.¹¹ Similarly, nuclear terminal deoxynucleotidyl transferase (TdT) is present in the leukemic cells in over 90% of ALL cases.¹² 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.⁹ However, several recent reports have studied CSF cells for the presence of TdT¹³⁻¹⁵ or cell surface antigens¹⁶ 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.^{17,18} 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

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contaminating peripheral blood containing lymphoblasts,^{19,20} samples containing red blood cell (RBC) (≥ 1 RBC per $100\times$ 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 studies¹⁸). 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.¹⁸ 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 provided an internal control for the nonspecific binding of spheres by cells since CD2 positive cells would not be expected to also be positive for CD10. MoAb was incubated with colored fluorescent polystyrene 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 containing 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 II; Shandon Elliot, Sewickley, PA) onto glass slides. The slides were either Wright-stained at this point or underwent fixation for TdT determination. The slides were examined under oil immersion at $100\times$ magnification using conventional or fluorescence microscopy (Olympus, model BH2-RFL). Slides were examined under conventional light to characterize cell morphology and TdT nuclear staining, and under blue or green fluorescence to enumerate binding with green spheres (anti-CD2) or red sphere (anti-CD10) binding. Cells bound with three or more spheres of a single color were counted as positive for the corresponding antigen. This criteria for cell surface antigen positivity correlates well with antigen positivity defined by other immunofluorescent techniques as has been reported by ourselves and other investigators using similar immunosphere labeling methods.^{18,21-25} Results were expressed as the percent of lymphocytes positive for a particular antigen per sample. Cells were not counted if a clump of spheres were bound to the cell at a single point, if the cells appeared damaged, or if three or more spheres of both colors were bound to the cells (as is the case with monocytes, damaged cells, and rare normal-appearing lymphocytes). Monocytes

and neutrophils were identified by morphology and immunosphere phagocytosis.

TdT testing. Slides to be tested for TdT were fixed in methanol at 4°C for 30 minutes and stored in desiccation jars at -20°C .²⁶ TdT testing was performed with a kit using a modified avidin-biotin peroxidase method (Sigma Diagnostics, St Louis, MO). After rehydration in phosphate buffered saline (PBS), the slides were incubated in 3% hydrogen peroxide to eliminate endogenous myeloperoxidase. The cells were then incubated in 10% sheep serum to block nonspecific antibody binding. After subsequent washes in PBS, the slides were incubated with a monoclonal mouse anti-TdT antibody (Sigma) for 30 minutes at 20°C . After washing with PBS, the slides were incubated for 30 minutes with a biotinylated sheep anti-mouse antibody. The slides were then washed with PBS, and incubated with streptavidin-peroxidase for 30 minutes, washed with PBS, and subsequently incubated with a Diamniobenzidine peroxidase substrate (Sigma) and counterstained with hematoxylin. The slides then had a coverslip applied and were examined as described above. The presence of TdT is identified by brown staining of the cell nucleus. Cells were not considered positive if the staining was limited to the cell membrane or cytoplasm. Negative and positive control 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 concurrent controls both 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 $8,700/\mu\text{L}$ (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 nonleukemic 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 counts²⁷ indicating little cell loss due to immunosphere typing. Of the 33 samples, 6 (18%) had cells with morphologic abnormalities which were either diagnostic (3 samples) or suspicious (3 samples) for malignancy. The

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.²⁸⁻³¹ 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 subjects with abnormal CSF cell morphology (50% to 88% CD10 positive cells per sample in the three samples with definite blasts and 4% to 9% CD10 positive cells in the three samples with suspicious blasts). However, the samples at diagnosis without morphologic abnormalities present also had an increase in CD10 positive cells (mean, 5.4% per sample). The difference between the mean CD10 positivity of the patients with morphologically normal CSF cells at diagnosis and the control patients was significant ($P = .001$). Perhaps a more clinically meaningful figure may be the proportion of patients having immunologically abnormal 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 with morphologically normal CSF at diagnosis had abnormal CSF immunophenotypes as opposed to 4 of 104 control patients ($P = .001$ by chi square). Therefore, of the entire group studied at diagnosis, morphologic or immunophenotypic abnormalities were present in 15 of 33 or 45% of the patients (28% to 64% within 95% confidence limits).

Because CSF cell surface antigen abnormalities could potentially be explained by the presence of CD10 on cells of neural origin³² or by the adsorption of free antigen onto normal lymphocytes, we studied a subset of samples for the simultaneous presence of surface CD10 and nuclear TdT. These results demonstrated that in 15 patients (with morphologically normal CSF) studied at diagnosis the mean percent-

age of TdT positive cells was 7.2% (range, 0% to 57%), and cells simultaneously expressing CALLA and TdT was 6.0% versus 0.05% in 28 controls ($P = .04$). A subset of 28 controls (9 off therapy and 19 on maintenance) were studied for three cell markers (CD10, CD2, and TdT). Six of 15 patients at diagnosis had elevated numbers (greater than 2 SD than the mean, or over 0.5%) of cells positive for both TdT and CD10 versus 2 of 28 controls ($P = .008$ by chi square).

Another explanation for the observed increase in CD10 positive cells at diagnosis is that, despite our attempts at censoring samples with visible RBC, peripheral blood leukemic cells could have nonetheless contaminated the CSF specimens during the collection process. If this were the case, it would be expected that CSF CD10 positive cells would be increased in those patients with the highest peripheral WBC blast counts (with greater numbers of potentially contaminating blasts present). Of the 25 patients in whom complete data are available, no significant correlation existed between CSF CD10 positive cells and peripheral blood WBC ($r = .145$).

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 cytopsin 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.⁸ To more easily compare the results of various treatment regimens, standardized criteria have been proposed for the diagnosis of CNS leukemia³³; 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 multivariate analysis.³⁴

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. Unlike some previous

Table 1. CSF Lymphocyte Immunophenotype Findings in Subjects With Normal CSF Cell Morphology

	Patient Group		<i>P</i>
	ALL at Diagnosis (n = 27)	Control (n = 104)	
CD 10 positive (%)	5.4 ± 1.1	0.8 ± 1.1	.001
CD 2 positive (%)	62.0 ± 2.2	73.0 ± 1.6	.02
TdT positive (%)	7.2 ± 1.7	0.8 ± 1.0	.05
TdT and CD 10 positive (%) (simultaneous)	6.0 ± 1.5	.05 ± 0.19	.04

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.

studies,^{13,14} 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.³⁷ 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.¹³ 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 leukemic 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.³⁸⁻⁴⁰ These previous reports, 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 of 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 Hooijkass 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.¹⁶ 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 al⁴¹ 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,⁸ and could potentially be useful in identifying groups of patients requiring more or less intensive presymptomatic CNS therapy.

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