Terminal Deoxynucleotidyl Transferase (TdT)-Positive Cells in Cerebrospinal Fluid and Development of Overt CNS Leukemia: A 5-Year Follow-up Study in 113 Children With a TdT-Positive Leukemia or Non-Hodgkin’s Lymphoma

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We investigated whether an indirect nuclear terminal deoxynucleotidyl transferase (TdT) immunofluorescence (IF) assay on single cells present in the cerebrospinal fluid (CSF) is more effective than conventional cytomorphology for early detection or exclusion of (minimal) meningeal leukemia infiltration in patients with a TdT+ malignancy. During a 5-year follow-up study, 1,661 consecutive CSF samples from 113 children with a TdT+ acute lymphoblastic leukemia (ALL) (n = 100), a TdT+ acute nonlymphoblastic leukemia (ANLL) (n = 8), or a TdT+ non-Hodgkin’s lymphoma (NHL) (n = 5) were analyzed. In 1,511 (91.9%) of 1,643 evaluable CSF samples, the positive and negative findings of both cytomorphology and the TdT-IF assay were concordant. In 47 (2.9%) samples from 28 patients, the cytomorphology was suspect while the TdT-IF assay was negative; follow-up as long as 58 months revealed no CNS leukemia in any patient. In 85 (5.2%) samples, cytomorphology was negative (n = 70) or suspect (n = 15) but TdT+ cells were detected. RBC contamination seriously hampered evaluation in 31 of these 85 samples. From the remaining 54 TdT+ samples from 29 patients, 40 samples preceded overt CNS leukemia in 20 patients. Two consecutive findings of TdT+ cells in the CSF were always followed by overt CNS leukemia. At initial diagnosis, 11 children had TdT+ cells in their RBC-free CSF. In one of these children, morphology was suspect; a repeated lumbar puncture was positive on both assays. Thus, initial CNS leukemia was diagnosed. In the other ten children, morphology was negative. In six of them, CNS leukemia was diagnosed 2 to 20 months later. In 32 other children examined at initial diagnosis, neither TdT+ cells nor blasts were observed in the CSF. In none of these patients was a CNS leukemia diagnosed after a follow-up of 2.5 to 57 months (median 24 months). In 207 control CSF samples from 58 children with TdT- oncologic, hematologic, or infectious diseases, no TdT+ cells could be detected. The TdT-IF assay is easy to perform and is a more reliable diagnostic tool for detection of CNS leukemia at an early stage than is cytomorphology. At initial diagnosis, the finding of TdT+ cells in a RBC-free CSF sample with a negative cytomorphology is highly predictive for development of overt CNS leukemia.

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TdT⁺ CELLS IN CEREBROSPINAL FLUID

surface marker covers all subtypes of ALL and some subtypes of ANLL and NHL as efficiently as does TdT.

To determine whether the TdT-IF assay improves detection or exclusion of (minimum) leukemic CNS infiltrates in patients with TdT⁺ malignancies, a longitudinal study was started in 1983. We report a 5-year follow-up study involving exclusion of (minimum) leukemic CNS infiltrates in observers.

Since the TdT-IF assay was considered experimental, other sites were considered. For calculation of the median follow-up, development of CNS leukemia, only patients who did not relapse at enlargement, no initial CNS leukemia were treated according to the national DCLSG protocol V, in use until November 1983 (A),47 or high-risk related to clinical characteristics at diagnosis. The children without leukemic BM were treated with a modified APO protocol (D). To a local protocol, in use until December 1987 (C). Children with initial WBC > 50 x 10⁹/L were treated according to a local protocol, in use until December 1987 (C). Children with mediastinal involvement and with >25% blasts or without a leukemic BM were treated with a modified APO protocol (D).

Induction treatment in protocols A, B, and C consisted of vincristine (VCR), prednisone, and l-asparaginase. A randomization for addition of daunorubicin was made in protocol A. Maintenance treatment consisted of 7-week cycles of 5 weeks of daily oral 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) alternating with 2 weeks of prednisone and 2 weekly injections of VCR for 2 years (A,B) or 3 years (C). In protocol B, prednisone was substituted by dexamethasone from December 1984 on. In protocol C, cyclophosphamide, once every fortnight, was added in the 6-MP/MTX period during the first year of maintenance treatment. In protocol D, induction treatment consisted of VCR, prednisone, and doxorubicin, followed by l-asparaginase. Maintenance treatment in this protocol consisted of pulses of high-dose prednisone, high-dose 6-MP, VCR, and doxorubicin every 3 weeks; five days of MTX was substituted for doxorubicin after the first 9 months.⁴⁶,⁴⁰

CNS prophylaxis for protocols A and C consisted of 25 Gy cranio-cervical irradiation plus five intrathecal injections of MTX (delivered in 2½ weeks) as soon as remission-induction treatment ended. In protocol C, six intrathecal injections with MTX, with a 7-week interval, were administered in addition during the first year of maintenance treatment.

In protocol B, CNS prophylaxis consisted of two intrathecal injections of MTX at weeks 2 and 4 of remission-induction treatment, followed by three 24-hour infusions of 2 gr MTX plus an intrathecal injection of MTX, delivered weekly during the first 3 weeks after the end of remission-induction treatment. During the first year of treatment, triple (MTX, cytarabine (ARA-C), and prednisone) therapy was administered intrathecally every 7 weeks (eight times).⁴⁸ In protocol D, the originally described CNS prophylaxis with cranial irradiation plus five intrathecal injections of MTX followed by an intrathecal injection of MTX once every 18 weeks was changed as follows: CNS prophylaxis consisted of three intrathecal injections of MTX plus ARA-C at weeks 3, 6, and 9 during remission-induction and consolidation treatment, followed by three 24-hour infusions, once every fortnight, of 2 g MTX plus intrathecal MTX and ARA-C followed by an intrathecal injection of MTX plus ARA-C every 6 weeks during 1 year (eight times).

Cell sampling and cytomorphology. CSF was obtained by lumbar puncture and collected in two plastic tubes, 2 to 5 mL each. One tube was processed in the diagnostic laboratory of the Subdivision of Hematology/Oncology within 30 minutes after sampling for cell counting and cytomorphology; the other was sent to the diagnostic laboratory of the Department of Immunology and was subjected to the TdT-IF assay within three hours after sampling. Cells were counted in a Fuchs-Rosenthal chamber. At least two cytocentrifuge preparations were made (0.5 mL each) with a Shandon-Elliott centrifuge (72 g, ten minutes) and stained with May-Grunwald-Giemsa or additional cytochemical stainings. Cytomorphology was considered positive when blasts were found that were unequivocally considered leukemic, irrespective of the CSF cell count. CSF samples that contained questionable blastlike cells or atypical (nonviral) lymphocytes were regarded as “suspect.”

TdT-IF assay. For the TdT-IF assay, CSF samples (2 to 5 mL) were centrifuged (300 g, five minutes). The cell pellets were resuspended to 100 µL, and two cytocentrifuge preparations were made (200 g, 3 minutes, Cytofuge, Nordic Immunological Laboratories, Tilburg, The Netherlands). These were air-dried, fixed in methanol (30 minutes, 4°C) and washed in phosphate-buffered saline (PBS), pH 7.8, for 15 minutes. One preparation was incubated with 15 µL optimally titrated rabbit anti-TdT antiserum; the second preparation, as a control, was incubated with 15 µL normal rabbit serum (moist chamber, 30 minutes, room temperature). After being washed in PBS, the preparations were incubated with 15 µL fluorescein conjugated goat anti-rabbit immunoglobulin antisera and washed again.⁵¹ The anti-TdT antiserum and the second-step antiserum were obtained from Bethesda Research Laboratories (Bethesda, MD) and from 1984 on from Supertechs (Bethesda, MD). The preparations were then mounted in glycerol PBS (9:1) containing p-phenylenediamine 1 mg/mL (BDH Chemicals, Poole, England) to prevent fading of fluorochrome, covered with a coverslip, and sealed with paraffin wax with crescin (BDH chemicals).³ Two cells expressing nuclear TdT were enumerated using Zeiss fluorescence microscopes equipped with phase-contrast facilities. A concentration effect (centrifugation of 2 to 5 mL CSF) might be the reason why we almost invariably detected cells on the TdT cytocentrifuge preparations, even when cell counts were low (<5/µL).
possible, 100 to 2,000 cells were screened. In 19% of cases, however, the low cell count of the CSF allowed screening of 10 to 100 cells only; 63% of the preparations contained 100 to 500 cells, whereas 18% contained >500 cells. Less than 1% of the preparations contained <10 cells. The percentage of TdT+ cells was calculated as the fraction of the number of nucleated cells, as determined by phase-contrast morphology. If any TdT+ cells were found in an atraumatic (RBC-free) spinal tap, the result of the TdT-IF assay was considered positive, irrespective of the total number of cells in the cytocentrifuge preparation or the CSF cell count.

RESULTS

Of 1,661 CSF samples available, 18 samples were non-evaluable for comparison between cytomorphology and the TdT-IF assay since it proved impossible to perform both examinations. In 1,643 samples from 113 children with a TdT+ acute leukemia or NHL, the correlation between cytomorphology and the TdT-IF assay could be examined. Findings are summarized in Table 1. In 88.0% of the 1,643 evaluable samples, both cytomorphology and the TdT-IF assay were positive; in 3.9% of the 1,643 samples, the results were both positive. The cytomorphology was suspect in 62 samples (3.8%). In 47 of these samples (2.9%) from 28 patients, no TdT+ cells were observed. No meningeal involvement has occurred in these patients during a median follow-up period of 26 months (range 1 to 58 months). In the remaining 15 (0.9%) cytologically suspect samples, TdT+ cells were observed. In 70 other samples (4.3%) in which no blasts were observed by cytomorphologic examination, TdT+ cells proved to be present (Table 1).

Table 1. Correlation Between Cytomorphology and TdT-IF Assay in 1,843 CSF Samples From 113 Patients With a TdT+ ALL (n = 100), a TdT+ ANLL (n = 8), or a TdT+ NHL (n = 5)

<table>
<thead>
<tr>
<th>TdT-IF Assay</th>
<th>Cytomorphology</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdT+ ALL</td>
<td>Negative</td>
<td>1,446 (88.0%)</td>
<td>70 (4.3%)</td>
<td>1,516 (92.3%)</td>
</tr>
<tr>
<td></td>
<td>Suspect</td>
<td>47 (2.9%)</td>
<td>15 (0.9%)</td>
<td>62 (3.8%)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>0 (0%)</td>
<td>65 (3.9%)</td>
<td>65 (3.9%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1,493 (90.9%)</td>
<td>150 (9.1%)</td>
<td>1,643 (100.0%)</td>
</tr>
</tbody>
</table>

The follow-up of the 49 ALL, one ANLL, and two NHL patients who contributed to the 85 TdT+ but cytomorphologically suspect CSF samples is summarized in Tables 2 and 3; Table 2 shows data of children with TdT+ samples at initial diagnosis; Table 3 shows data of those with a first TdT+ sample during maintenance therapy or after cessation of treatment. Data are subdivided in CSF samples with or without RBC contamination (Tables 2 and 3). The 31 RBC-contaminated CSF samples (18 at diagnosis and 13 during follow-up) were considered nonassessable. From the 54 RBC-free TdT+ samples, 11 were obtained at initial diagnosis from nine ALL and two T-NHL patients (Table 2). The percentages of TdT+ cells ranged from 0.3% to 70%. In one child, the CSF was cytologically suspect, and a spinal tap repeated after five days contained both TdT+ cells and leukemic blasts according to cytomorphologic criteria. Thus, initial CNS leukemia was diagnosed. In the other ten patients, a second CSF sample obtained 2 to 3 weeks later, during the systemic induction treatment, did not contain TdT+ cells anymore. However, six of the ten patients (five ALL and one T-NHL) developed a CNS leukemia as diagnosed by cytomorphology 2 to 20 months later. The other four patients did not develop a CNS leukemia during a median follow-up of 38 months (range 31 to 55 months). In 32 patients without TdT+ cells in their RBC-free CSF samples at diagnosis, no CNS leukemia was diagnosed during a median follow-up of 24 months (range 2.5 to 57 months).

The other 43 RBC-free TdT+ CSF samples from 17 ALL, one ANLL, and two T-NHL patients were obtained during follow-up on or off therapy (Table 3). Nine patients (ten samples) did not develop CNS leukemia; five of them (six samples), however, relapsed in the BM and accordingly received reinduction therapy, including CNS prophylaxis. Two samples from two patients were obtained in the last phase of successful CNS leukemia reinduction treatment and represented the last positive finding in a series of several TdT+ samples in which cytomorphology was already negative. One of the remaining two samples was positive during CNS prophylaxis, and the other was positive during maintenance therapy. These two patients did not develop CNS leukemia at 48 and 46 months of follow-up, respectively. In 13 patients (33 samples), findings of TdT+ cells in the CSF were followed by a diagnosis of overt CNS leukemia within a median follow-up of 1.5 months (range 0.5 to 24 months). In our series, two consecutive findings of TdT+ cells in the CSF were always followed by overt CNS leukemia.

TdT+ cells were never detected in 207 RBC-free CSF samples from 58 patients with TdT+ leukemia or NHL or with other hematologic, oncologic, or infectious diseases. Moreover, several patients in our leukemia and NHL follow-up study had episodes with bacterial or viral meningitis, sometimes paralleled with high CSF cell counts. However, neither were TdT+ cells noted in the CSF of patients with these infections.

DISCUSSION

The present study was undertaken to investigate (a) whether the presence of TdT+ cells in the CSF, as demonstrated by TdT-IF assay, would be helpful for early diagnosis of CNS leukemia, and (b) whether the absence of TdT+ cells would strongly argue in favor of the absence of meningeal involvement. The result would have direct implications for treatment.

Our data indicate that all CNS leukemias diagnosed on clinical and cytomorphologic criteria were always confirmed by TdT-IF assay, resulting in a 100% sensitivity. In addition, we were able to detect TdT+ cells before overt CNS leukemia according to cytomorphologic criteria could be diagnosed (Tables 2 and 3). We further investigated whether the presence of TdT+ cells (even in low numbers) is indicative of meningeal involvement. TdT+ cells do not belong to the RBC-free CSF samples at diagnosis, no CNS leukemia was diagnosed during a median follow-up of 24 months (range 2.5 to 57 months).

The present study confirms the findings of other investigators35 that TdT+ cells do not occur in the CSF of nonleukemic patients. We further investigated the CSF of
Table 2. Follow-up of 27 ALL and Two NHL Patients With TdT+ Cells in CSF at Initial Diagnosis, but With Negative or Suspect CSF Cytomorphology (n = 29 Samples)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>CSF TdT-IF Sample</th>
<th>CSF TdT-IF Interpretation</th>
<th>Clinical Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RBC, negative cytology</td>
<td>1 M</td>
<td>7</td>
<td>62.0</td>
<td>75</td>
<td>83 T-ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>2 M</td>
<td>2</td>
<td>34.9</td>
<td>62</td>
<td>95 Common ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>3 M</td>
<td>3</td>
<td>10.3</td>
<td>37</td>
<td>98 Common ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>4 F</td>
<td>3</td>
<td>15.1</td>
<td>58</td>
<td>93 Pre-B ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>5 M</td>
<td>15</td>
<td>3.1</td>
<td>60</td>
<td>68 Common ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>6 F</td>
<td>5</td>
<td>266.0</td>
<td>73</td>
<td>92 T-ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>7 F</td>
<td>4</td>
<td>5.1</td>
<td>27</td>
<td>85 Pre-B ALL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>8 M</td>
<td>13</td>
<td>7.0</td>
<td>0</td>
<td>0 T-NHL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>9 F</td>
<td>8</td>
<td>5.9</td>
<td>0</td>
<td>3 T-NHL</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>10 F</td>
<td>5</td>
<td>9.9</td>
<td>0</td>
<td>3 T-NHL</td>
</tr>
</tbody>
</table>

Table 3. Evaluation of 29 Patients With TdT+ Cells in CSF During Follow-up but With a Negative or Suspect CSF Cytomorphology (n = 56 Samples)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Samples</th>
<th>TdT+ Cells</th>
<th>Interpretation</th>
<th>Clinical Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RBC, negative cytology</td>
<td>13</td>
<td>33</td>
<td>0.1-7.2</td>
<td>Positive 2 (0.5-24)</td>
</tr>
<tr>
<td>No RBC, suspect cytology</td>
<td>10</td>
<td>60</td>
<td>0.2-3.0</td>
<td>Positive 4 (1-48+)</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>13</td>
<td>0.01-1.4</td>
<td>Not assessable§ 7 (4-56+)</td>
</tr>
</tbody>
</table>

*Four patients are represented in more than one category.
†Five patients developed a BM relapse.
‡Three patients developed a BM relapse.
§Not assessable due to RBC contamination.
clear. All ten children with TdT+ cells in the CSF at initial diagnosis received comparable CNS prophylaxis. At present, we must assume that the CNS prophylaxis was able to prevent or eliminate meningeal leukemia in these four patients. However, CNS leukemia can still occur many years after initial diagnosis; thus, the period of follow-up may still be too short. Nevertheless, our data indicate that a positive TdT-IF assay in the first RBC-free CSF sample of untreated patients selects for those at high risk of developing overt CNS leukemia according to standard criteria. Thus, we believe that the finding of TdT+ cells in a RBC-free CSF sample at diagnosis should be taken into account for a diagnosis of initial CNS leukemia and as a consequence should lead to a more intensive CNS treatment.

Concerning the patients under maintenance treatment or treated for CNS leukemia, two consecutive findings of TdT+ cells in RBC-free CSF samples without cytomorphologically detectable leukemic blasts were always followed by a cytomorphologically confirmed CNS leukemia. Incidental findings of TdT+ cells in the CSF from such patients did not always result in overt CNS leukemia as a first event. Therapy, especially the therapy administered as a consequence of relapses at other sites than the CNS, might have prevented development of overt meningeal leukemia in these patients. However, a first finding of TdT+ cells in the CSF from patients off therapy was always followed by a second finding. Subsequently, overt CNS leukemia always resulted, indicating that a single finding of TdT+ cells in a CSF sample from patients after cessation of treatment might well be sufficient for the diagnosis of CNS leukemia to relapse.

In our experience and that of other researchers, the TdT-IF assay can be performed easily, even when low numbers of cells are present in the CSF. The TdT-IF assay proved highly sensitive for evaluation of CSF samples from patients with TdT+ leukemia or NHL. This assay contributes to early detection as well as exclusion of CNS leukemia. In our opinion, it is a most reliable tool for diagnosing CNS leukemia in patients with TdT+ malignancies. Moreover, it also offers the possibility for selection at diagnosis of patients at high risk of developing overt CNS leukemia. Therefore, the diagnostic criteria for presence or absence of CNS leukemia should include the findings of the TdT-IF assay.

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