Shedding of CD9 Antigen Into Cerebrospinal Fluid by Acute Lymphoblastic Leukemia Cells

By Yoshihiro Komada, Hitoshi Ochiai, Ken Shimizu, Eiichi Azuma, Hitoshi Kamiya, and Minoru Sakurai

The accurate identification of small numbers of leukemic cells in the cerebrospinal fluid (CSF) presents a diagnostic problem in the treatment of acute lymphoblastic leukemia (ALL). We demonstrated that soluble CD9 antigen was shed into CSF obtained from children with ALL, using enzyme-linked immunosorbent assay (ELISA), which used the activity of CD9 antigen to bind the Ricinus communis agglutinin (RCA1) and a monoclonal antibody, SJ-9A4, simultaneously. Using RCA1/SJ-9A4 ELISA, CD9 antigen was detectable in CSF but not in plasma from 12 cases of CD9+ ALL in central nervous system (CNS) relapse. However, CD9 antigen was not released into CSF from 11 cases of CD9- ALL with CNS involvement, 136 cases of CD9+ ALL in complete remission (CR). 29 cases of CD9- ALL in CR, or 21 cases of aseptic meningitis. Interestingly, the levels of CD9 antigen were elevated in CSF from 7 of 10 CD9+ ALL patients without cytologically proven CNS involvement at diagnosis, with subsequent return to undetectable levels after initial induction chemotherapy was begun. In addition, serial quantitative analysis of CSF from a 5-year-old boy with CD9+ ALL in CNS relapse showed that levels of CD9 antigen correlated well with the number of leukemic cells in CSF. Serial quantitative analysis of CD9 antigen in CSF could be useful to detect the proliferation of residual leukemic cells before the clinical manifestation.

The prognosis for children with acute lymphoblastic leukemia (ALL) has been dramatically improved over the last few decades. However, relapses in the central nervous system (CNS) remain a difficult problem despite prophylactic CNS treatment. Current ALL treatment protocols monitor cerebrospinal fluid (CSF) samples for the development of meningeal leukemia. Overt CNS relapse is specifically detected in CSF from CD9+ ALL patients with CNS involvement. Shedding of CD9 antigen by leukemic cells may enable the clinical monitoring of leukemic cell burden in the CNS.

MATERIALS AND METHODS

CSF. Samples of CSF were obtained from ALL children being treated in the Pediatric Oncology Clinic, Mie University Hospital, Japan, and from patients with aseptic meningitis. Most children with ALL were being treated on current Tokai Pediatric Oncology Group Treatment Protocols, and samples were obtained at times specified by those protocols. In addition, serial samples of CSF were also collected at the time of diagnosis and during initial induction chemotherapy, which consisted of vincristine (2.0 mg/m2 weekly) and prednisone (120 mg/m2 daily). Ordinarily, 3 mL of CSF was obtained from each patient and was immediately put on ice. Two milliliters were used for cell count and cytocentrifuge examination for differential. In this study, CNS involvement was defined as greater than 5 mononuclear leukocytes per microliter of CSF with morphologically unequivocal lymphoblasts in cytocentrifuge samples. The remaining 1 mL was rendered cell-free by centrifugation at 540g for 10 minutes at 4°C immediately after collection of CSF specimen. This was further ultracentrifuged at 100,000g for 1 hour at 4°C. The supernatant was then cryopreserved at −20°C until tested for soluble CD9 antigen using ELISA.

MoAb used in this study. MoAb against CD9 antigen, SJ-9A4 (immunglobulin G1, [IgG1], ) was used in this study. The characterization of SJ-9A4 was previously described. Matched concentrations of mouse myeloma protein of the same Ig isotype were used as negative controls.

ELISA for soluble CD9 antigen. This assay depends on the ability of CD9 glycoprotein antigen to bind Ricinus communis agglutinin (RCA1; E-Y Laboratories, San Mateo, CA) via an oligosaccharide chain with a terminal D-galactose and to the appropriate MoAb. Microtiter wells (Immulon 1; Dynatech, Alexandria, VA) were coated with 0.025 mg of RCA1 in 100 μL of 0.045
mol/L NaHCO₃, pH 9.6 (coating buffer). The plates were incubated overnight at 4°C and washed with 0.01 mol/L phosphate-buffered saline (PBS) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co, St Louis, MO) (washing buffer).

One hundred microliter samples for detection of soluble CD9 antigen were added to each well and incubated overnight at 37°C. After wells were washed three times with washing buffer, 100 μL of SJ-9A4 or negative control (1:4 diluted supernatant medium) was added to each well. The wells were incubated for 1 hour at room temperature and then washed three times. Next, 100 μL of rabbit anti-mouse Ig (RamIg; Cappel Laboratories, West Chester, PA) (1:50 diluted with PBS) was added and allowed to incubate for 1 hour at room temperature. After washing, 100 μL of alkaline phosphatase, mouse anti-alkaline phosphatase MoAb (APAAP; Kyowa Medex, Tokyo, Japan) (1:500 diluted with PBS) was added to each well. The wells were incubated for an additional hour at room temperature and washed three times with washing buffer. Finally, 100 μL of substrate buffer (97 mL of diethanolamine, 0.1 g of MgCl₂·6H₂O, and 0.2 g of sodium azide per liter, pH 9.8) containing 0.01% p-nitrophenyl phosphate disodium (Sigma) was added and the color was allowed to develop for 30 minutes at 37°C. Absorption of light at 405 nm (OD₄₀₅) was determined on MICROELISA Auto Reader MR 580 (Dynatech). Results were plotted as the level of CD9 antigen detected (OD₄₀₅ bound by SJ-9A4/OD₄₀₅ bound by negative control).

**Plasma extraction.** Peripheral blood was obtained from ALL patients with CNS involvement at the same time that CSF was collected. To detect soluble CD9 antigen in patient plasma, it was necessary to optimize the fractionation procedure because of the presence of a high concentration of glycoproteins that could bind to RCA1. This inhibitory effect could be alleviated by fractionation of the sample as previously described.* In brief, peripheral blood from patients with ALL at the time of CNS relapse was anticoagulated with EDTA and immediately centrifuged at 200g for 15 minutes at 4°C. Plasma was collected and precipitated with 45% saturated ammonium sulfate (SAS). The precipitate was washed with 45% SAS and dissolved in 35% SAS. The precipitate was discarded and the supernatant was dialyzed exhaustively against 0.01 mol/L PBS. This fraction was then passed over an immunoadsorbent column of rabbit anti-human serum protein coupled to Sepharose-4B (DAKO Corp, Santa Barbara, CA). The resulting supernatant was tested in the RCA1/SJ-9A4 ELISA. The rabbit anti-human serum protein reagent did not crossreact with CD9 antigen, and the percent yield of CD9 antigen obtained after plasma fractionation was more than 80%, as previously reported.*

**Phenotypic analysis.** Leukemic cells for cell surface antigen testing were obtained from peripheral blood, bone marrow aspirates, and CSF. Peripheral blood and bone marrow aspirates were collected from patients at diagnosis and the mononuclear cells were isolated by Ficoll-Hypaque (Sigma) centrifugation. Leukemic cells in CSF from patients in CNS relapse were separated by centrifugation at 200g for 10 minutes. In all instances, the cell suspensions contained more than 90% leukemic cells as assessed by May-Giemsa morphology. The cells were characterized by indirect immunofluorescence and the leukemic cell surface phenotype was defined as previously reported.**

**RESULTS**

**Specificity of ELISA for soluble CD9 antigen in CSF.** The assay system uses RCA1 adsorbed to polystyrene wells to bind soluble CD9 antigen on the solid phase. The amount of CD9 antigen bound is then quantitated by its ability to combine with SJ-9A4 MoAb. The amount of MoAb bound was determined by the addition of RamIg and APAAP complex. When saturating amounts of SJ-9A4, RamIg, and APAAP complex were added, the specific binding was directly proportional to the amount of CD9 antigen bound to the solid-phase RCA1.**

The sample of CSF obtained from a 5-year-old boy (T.T.) having positive blasts for CD9 antigen at the time of CNS relapse contained detectable CD9 antigen. However, no binding occurred when wells were treated with coating buffer or bovine serum albumin (RIA grade; Sigma) in coating buffer. When SJ-9A4 was replaced by a control myeloma protein of the same Ig isotype, no binding occurred. The presence of 4% D-galactose gave complete and specific inhibition of binding of CD9 antigen to RCA1. Matched concentration of D-glucose and N-acetyl galactosamine had no effect on binding.

**Detection of CD9 antigen in CSF.** Samples of CSF were obtained from 148 patients with CD9+ ALL at the time of CNS relapse (12 cases) or in complete remission (CR) (136 cases). Control samples were from 40 patients with CD9- ALL at the time of CNS relapse (11 cases) or in complete remission (CR) (29 cases), and from 21 patients with aseptic meningitis. All samples were ultracentrifuged and assayed for CD9 antigen activity by ELISA (Fig 1). Soluble CD9 antigen was specifically detected in CSF from all 12 patients with CD9+ ALL at the time of CNS relapse. Phenotypic
analysis showed that blast cells in CSF from these patients were positive for CD9 antigen. However, soluble CD9 antigen was undetectable in the fractionated plasma from these patients at the time of CNS relapse (Table 1). The levels of CD9 antigen were independent of blast cell count in CSF. Ultracentrifugation (100,000g for 1 hour) had no effect on soluble CD9 antigen present in CSF. Samples from 136 patients with CD9+ ALL in CR contained no detectable CD9 antigen. None of the patients with CD9- ALL or aseptic meningitis showed significant levels of CD9 antigen.

**Serial quantitation of soluble CD9 antigen in CSF.** The CSF from 10 patients with CD9+ ALL at the time of diagnosis was assayed for CD9 antigen activity by ELISA (Fig 2). Seven of 10 cases showed elevated levels of CD9 antigen in CSF obtained before induction chemotherapy was initiated. It is noteworthy that these 10 patients were clinically and cytologically free from CNS involvement at onset, and no quantitative correlation was found between the levels of CD9 antigen activity in CSF and blast cell count in peripheral blood (Table 2). Samples obtained during induction chemotherapy contained a decreasing amount of soluble CD9 antigen, with subsequent return to undetectable levels. Two cases of CD9- ALL showed no detectable CD9 antigen activity in CSF at the time of diagnosis and in the early period of induction chemotherapy.

In addition, samples of CSF were serially obtained from a patient (T.T.) with CNS leukemia (Fig 3). Soluble CD9 antigen activity was not detected before CNS relapse. However, significant CD9 antigen activity was detectable at the time of CNS relapse and subsequently decreased to lower levels after intrathecal medication was initiated. The level of CD9 antigen, as shown in Fig 3, correlated well with the number of blast cells in CSF.

**DISCUSSION**

In this study we have demonstrated that soluble CD9 antigen was released into CSF by CD9+ leukemic lymphoblasts. Furthermore, we have studied CD9 antigen levels in CSF obtained from ALL patients without CNS involvement at the time of diagnosis and have found that CD9 antigen activity was elevated in 7 of 10 patients with CD9+ ALL.

The release of glycoproteins from the surface membrane into the extracellular milieu is an active process known as shedding. This process is accelerated in activated and malignant cells in relation to normal counterpart cells. We have previously described the development of RCA1/SJ-9A4 ELISA for detecting soluble CD9 antigen from ALL cell lines in vitro. Extracts from as few as 5 x 10⁶ cells of a lymphoblastoid cell line, NALM-1, contained detectable levels of CD9 antigen. Saturation was achieved when extracts from 1 x 10⁶ cells were assayed. This system has been designed in such a way that the release of CD9 antigen from the surface of growing cells is not confused with the sloughing of membrane fragments by dividing or dying cells. Soluble CD9 antigen shedding was inhibited when cells were maintained at 4°C in vitro, indicating it to be an active process. In cultures that were serially monitored for CD9 antigen release, it was shown that most of it occurred during the early exponential growth phase, and only a small increment was associated with cell death.

The in vitro findings were corroborated by the presence of detectable CD9 antigen levels in CSF from ALL patients with CD9+ blasts. All 12 patients with CD9+ ALL studied had markedly elevated levels at the time of CNS relapse. Control CSF from ALL patients in CR, CD9- ALL patients in CNS relapse and patients with aseptic meningitis contained no detectable CD9 antigen activity. All CSF samples were immediately put on ice and rendered cell-free by centrifugation at 4°C. Therefore, it is unlikely that the mechanism for release of CD9 antigen into CSF is an artifact due to exfoliation from lymphoblasts in CSF samples stored in our clinical laboratory. It is possible that CD9 antigen in

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**Table 1. Detection of Soluble CD9 Antigen in CSF and Plasma From Patients With CD9+ ALL With CNS Involvement**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Mononuclear Cells in CSF (x10⁶)</th>
<th>% CD9 in Cells in CSF</th>
<th>Soluble CD9* in CSF</th>
<th>Soluble CD9 In Plasma</th>
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<tbody>
<tr>
<td>1</td>
<td>1.338</td>
<td>90</td>
<td>1.403</td>
<td>0.043</td>
</tr>
<tr>
<td>2</td>
<td>1.674</td>
<td>97</td>
<td>1.364</td>
<td>0.056</td>
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<td>3</td>
<td>1.200</td>
<td>83</td>
<td>1.296</td>
<td>0.073</td>
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<tr>
<td>4</td>
<td>4.964</td>
<td>92</td>
<td>0.898</td>
<td>0.042</td>
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<td>5</td>
<td>991</td>
<td>58</td>
<td>0.685</td>
<td>0.049</td>
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<tr>
<td>6</td>
<td>1.090</td>
<td>72</td>
<td>0.561</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>2.200</td>
<td>93</td>
<td>0.537</td>
<td>0.066</td>
</tr>
<tr>
<td>8</td>
<td>933</td>
<td>52</td>
<td>0.410</td>
<td>0.082</td>
</tr>
<tr>
<td>9</td>
<td>640</td>
<td>93</td>
<td>0.351</td>
<td>0.071</td>
</tr>
<tr>
<td>10</td>
<td>2.640</td>
<td>95</td>
<td>0.298</td>
<td>0.091</td>
</tr>
<tr>
<td>11</td>
<td>1.160</td>
<td>90</td>
<td>0.227</td>
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</tr>
<tr>
<td>12</td>
<td>988</td>
<td>88</td>
<td>0.208</td>
<td>0.068</td>
</tr>
</tbody>
</table>

*Abbreviation: ND, not determined.*

*Assay for soluble CD9 antigen was performed as described in Materials and Methods. Results were expressed as the level of CD9 antigen detected (OD₄₅₅).
CSF might be shed from lymphoblasts in blood rather than CSF. However, soluble CD9 antigen was undetectable in the fractionated plasma from ALL patients at the time of CNS relapse. Because the rabbit anti-human serum protein reagent did not crossreact with CD9 antigen, the plasma fractionation method removed very little CD9 antigen. Our previous study demonstrated that soluble CD9 antigen was detected in plasma from ALL patients with CD9+ blasts at the time of diagnosis, but the plasma samples obtained during CR were all negative. Because the patients with hematologic relapse were excluded from the present study, we concluded that soluble CD9 antigen in CSF would be shed from lymphoblasts in CSF in vivo.

We also found that serial monitoring of CD9 antigen activity in CSF from a 5-year-old boy in CNS relapse could provide an objective means of estimating leukemic burden. However, the levels of CD9 antigen shed into CSF were independent of blast cell count in 12 ALL patients with CNS involvement, as shown in Table 1. The previous studies showed that the levels of both CD9 antigen and CD10 antigen released into extracellular medium were also quite different among lymphoblastoid cell lines, and furthermore demonstrated that most of CD9 antigen shedding occurred during the early exponential growth phase. The amount of CD9 antigen shed might be dependent on proliferative activity of lymphoblasts and the number of blast cells in exponential growth phase, but not on total blast cell count. Because no positives were noted in 136 samples in CR, it was suggested that residual dormant lymphoblasts could not shed significant amounts of CD9 antigen in vivo. Serial quantitation of CD9 antigen shedding may be useful in detecting the proliferation of the residual leukemic cells before the clinical manifestation of relapse, but may not be sensitive enough to monitor the residual dormant leukemic cell burden during CR.

In most patients treated with modern chemotherapy, the diagnosis of CNS leukemia is made microscopically. The incidence of CNS leukemia at diagnosis depends partly on the diagnostic criteria used. If only a single lumbar puncture is performed, the chance of detecting CNS leukemia is less than if serial punctures are performed. However, if the second or third lumbar puncture is performed after initial induction chemotherapy is initiated, the likelihood of detecting microscopic CNS leukemia is decreased, because agents such as steroids cross the blood-brain barrier. In this study we showed the elevated levels of CD9 antigen activity in CSF from 7 of 10 CD9+ ALL patients at diagnosis who did not present with any clinical evidence of CNS leukemia. Subsequently, after induction chemotherapy was initiated, CD9 antigen activity in CSF returned to undetectable levels. These results suggested that quantitation of CD9 antigen shedding could be a sensitive and objective diagnostic method of detecting leukemic cells in CSF, although the accurate incidence of CD9 antigen shedding into CSF could not be estimated due to the small number of samples tested. It might be possible that CD9 antigen was shed into plasma by leukemic lymphoblasts and afterward transferred into CSF. The presence of CD9 antigen in CSF may result from the passive transfer of same antigen molecules, although no correlation is evidenced between the level of CD9 antigen in CSF and peripheral blast cell count. Because samples of CSF were immediately put on ice and rendered cell-free, it is unlikely that contaminating peripheral blood lymphoblasts shed CD9 antigen into CSF. However, CD9 antigen was not expressed on 20% of non-T, non-B ALL and 75% of T-cell ALL. This possible limitation of RCA/SJ-9A4 ELISA should be taken into consideration. Leukemic children who present with clinical evidence of CNS leukemia have a worse prognosis than those without this feature, and CNS irradiation should be included as a component of therapy for

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**Table 2. Detection of Soluble CD9 Antigen in CSF From Patients With CD9+ ALL at Diagnosis**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>WBC (x 10^9/L)</th>
<th>% Circulating Blasts</th>
<th>Soluble CD9 in CSF*</th>
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<tbody>
<tr>
<td>1</td>
<td>5.8</td>
<td>16</td>
<td>0.481</td>
</tr>
<tr>
<td>2</td>
<td>26.1</td>
<td>65</td>
<td>0.378</td>
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<tr>
<td>3</td>
<td>3.2</td>
<td>28</td>
<td>0.274</td>
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<tr>
<td>4</td>
<td>23.0</td>
<td>55</td>
<td>0.263</td>
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<tr>
<td>5</td>
<td>3.9</td>
<td>29</td>
<td>0.248</td>
</tr>
<tr>
<td>6</td>
<td>253.0</td>
<td>95</td>
<td>0.210</td>
</tr>
<tr>
<td>7</td>
<td>17.7</td>
<td>93</td>
<td>0.206</td>
</tr>
<tr>
<td>8</td>
<td>5.2</td>
<td>0</td>
<td>0.089</td>
</tr>
<tr>
<td>9</td>
<td>7.0</td>
<td>59</td>
<td>0.080</td>
</tr>
<tr>
<td>10</td>
<td>3.8</td>
<td>70</td>
<td>0.075</td>
</tr>
</tbody>
</table>

*Abbreviation: WBC, white blood cells.

*Assay for soluble CD9 antigen in CSF was performed as described in Materials and Methods. Results were expressed as the levels of CD9 antigen detected (OD405).*
all patients with overt CNS leukemia at diagnosis.\textsuperscript{4,24,25} The clinical relevance of the elevated CD9 antigen levels in CSF is presently unknown.

In summary, we have demonstrated that soluble CD9 antigen is shed by leukemic lymphoblasts in CSF. Prospective study is now in progress to determine whether serial quantitation of CD9 antigen in CSF is useful for early recognition of CNS leukemia and to clarify the clinical relevance of elevated CD9 antigen level at the time of diagnosis.

REFERENCES


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Shedding of CD9 antigen into cerebrospinal fluid by acute lymphoblastic leukemia cells

Y Komada, H Ochiai, K Shimizu, E Azuma, H Kamiya and M Sakurai