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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 (Ram Ig; 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 phosphate, mouse anti-alkaline phosphatase MoAb (APAAP; Kyowa Medex, Tokyo, Japan) (1:50 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."¹⁶ In brief, peripheral blood from patients with ALL at the time of CNS relapse was anticoagulated with EDTA and immediately centrifuged at 800g 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 immunosorbent 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."¹⁶

**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."¹⁶

**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 Ram Ig and APAAP complex. When saturating amounts of SJ-9A4, Ram Ig, and APAAP complex were added, the specific binding was directly proportional to the amount of CD9 antigen bound to the solid-phase RCA1."¹⁵ ¹⁹

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

![Fig 1. Detection of soluble CD9 antigen in CSF from children with ALL. Samples of CSF were ultracentrifuged and assayed for CD9 antigen activity using RCA1/SJ-9A4 ELISA. Results were plotted as the level of CD9 antigen detected. **, Mean ± 2 SD.](image-url)
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.12 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, NAL-M-1, contained detectable levels of CD9 antigen. Saturation was achieved when extracts from 1 x 10⁹ cells were assayed.19 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.16,19

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⁺ Cells in CSF</th>
<th>Soluble CD9 Antigen (OD₅₄₄) in CSF</th>
<th>Soluble CD9 Antigen (OD₅₄₄) in Plasma</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>3</td>
<td>1.200</td>
<td>83</td>
<td>1.296</td>
<td>0.073</td>
</tr>
<tr>
<td>4</td>
<td>4.964</td>
<td>92</td>
<td>0.898</td>
<td>0.042</td>
</tr>
<tr>
<td>5</td>
<td>991</td>
<td>58</td>
<td>0.855</td>
<td>0.045</td>
</tr>
<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>
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<td>8</td>
<td>933</td>
<td>52</td>
<td>0.410</td>
<td>0.082</td>
</tr>
<tr>
<td>9</td>
<td>892</td>
<td>95</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>
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<td>90</td>
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<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 (OD₅₄₄).
SHEDDING OF CD9 ANTIGEN

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>WBC x 10⁹/L</th>
<th>% Circulating Blasts</th>
<th>Soluble CD9 in CSF*</th>
</tr>
</thead>
<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>
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<tr>
<td>3</td>
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<td>28</td>
<td>0.274</td>
</tr>
<tr>
<td>4</td>
<td>23.0</td>
<td>55</td>
<td>0.263</td>
</tr>
<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 (OD₄₅₀).

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 RCAI/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...
all patients with overt CNS leukemia at diagnosis. 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

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