A B-Lymphoma Cell Line That Forms Rosettes With Neuraminidase-Treated Sheep Erythrocytes Through Monoclonal Surface Immunoglobulin

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Undifferentiated lymphoma from a 39-yr-old female became serially xenotransplantable to preirradiated nude mice. The tumor cells (KT) possessed a monoclonal surface immunoglobulin (Slg, μ, κ) and formed rosettes with neuraminidase-treated sheep erythrocytes (SEN). Precise characterizations of the SEN rosette, however, revealed the following facts: (1) Neuraminidase-untreated or 2-aminoethylisothiuronium bromide (AET) treated sheep erythrocytes were not bound to the KT cells. (2) SEN rosettes on the KT cells did not show a temperature dependency. (3) Neuraminidase-treated erythrocytes from man, horse, mouse, and rabbit were not bound to the KT cells. (4) Preincubation of the KT cells with antipolyvalent immunoglobulin or anti-κ-chain serum abolished the SEN rosette formation. (5) Trypsinization decreased both SEN rosettes and Slg on the KT cells. (6) SEN rosettes on the KT cells were too loose to be separated from nonrosetting cells by a Percoll gradient centrifugation method. Summarizing these results, the monoclonal Slg on the KT cells recognized sheep erythrocyte antigen(s) that were exposed only after the neuraminidase treatment. Therefore, this was considered to be a case with peculiar B-lymphoma cells that bound SEN through their Slg.

It is well known that lymphocytes can be subdivided into two major subtypes: T and B cells. However, in rare instances, phenotypic manifestations on T and B lymphocytes can be very confusing.

In this article, we report a case of malignant lymphoma that became serially transplantable to preirradiated nude mice and showed both monoclonal Slg (Ig μ, κ) and receptors for neuraminidase-treated sheep erythrocytes (SEN) on its cell surfaces. These markers were mostly unchanged following serial transplantations to nude mice. With precise characterizations of the surface receptors, we have revealed the fact that this lymphoma is composed of peculiar neoplastic B cells that bind SEN through their monoclonal Slg.

MATERIALS AND METHODS

Patient

A 39-yr-old female was admitted to Keio University Hospital in January 1979 with complaints of weight loss, abdominal distension, and dyspnea of 3-mo duration. Since the cytologic examination of ascites revealed malignant lymphoma, combined chemotherapy was administered. Cervical lymphadenopathy appeared in March 1979, and a biopsy confirmed the diagnosis. She expired in November 1979. Neither cold agglutinin activity nor the monoclonal immunoglobulin peak were detected in the serum throughout her illness.

Postmortem examination revealed generalized lymph node swelling, massive peritoneal disseminations and patchy intestinal invasions by lymphoma cells. Their slight to moderate involvements of the lung, kidney, and pancreas were also noted. Infiltration into the liver, spleen, and bone marrow was absent.

Nude Mice

Female BALB/c nude mice weighing 20–25 g were fed in a SPF condition. The mice were exposed to whole body irradiation (400 R) 3 days before transplantation trials. Blocks of fresh lymphoma tissue were inserted with a trocar into the subcutis of nude mice.

Surface Marker Studies

A part of fresh lymphoma tissues was minced by scissors, filtered through mesh, and washed 3 times with phosphate-buffered saline (PBS), pH 7.4. Receptors for neuraminidase-treated sheep erythrocytes (SEN), those for the Fc portion of IgG or IgM (OxEA IgG or OxEA IgM), and those for the human or murine C3 component of complements (OxEAch or OxEACm) were detected by the methods described previously. Cells surrounded by more than 3 indicator erythrocytes were scored as positive. Surface immunoglobulins were detected by incubation for 30 min at 4°C with fluorescein isothiocyanate (FITC)-labeled rabbit IgG against human immunoglobulins, monospecific for γ, α, μ, δ, ε, κ, and λ determinants (DAKO) with added sodium azide (0.02%) in the solution. FITC-labeled F(ab')2 fragments of rabbit IgG against human μ-chain were also used to rule out the reactivity through the Fc receptors. Complement-dependent cytotoxicity tests were done by using anti-T-serum prepared by immunization with MOLT-4F cells and by using anti-"Ia" serum prepared by immunization with Raji cells. An immunofluorescence study using anti-"Ia" serum was also performed.

Characterization of SEN Rosettes

For characterization of SEN rosettes, the following experiments were performed. (1) The tumor cells (KT) or normal peripheral blood lymphocytes (PBL) were incubated with neuraminidase-untreated native SE from 3 lots. (2) SE were treated with CI perflingens neuraminidase (Sigma, St. Louis, Mo., type VI) in the presence of 20 mM EDTA, which is known to inhibit the enzyme activity. Instead of the enzyme treatment, SE were treated with 2-aminoethylisothiuronium bromide (AET, Sigma), which enhances the classical SE rosette formation. (4) The KT cells or normal PBL were treated with AET or neuraminidase under the same conditions as SE. (5) Neuraminidase-treated or untreated erythrocytes from other species, such as man (AB type), horse, mouse, and rabbit, were
used as indicator cells. (6) The KT cells were incubated with SEn only for 15 min at 37°C, 20°C, or 4°C. (7) The KT cells were preincubated with anti-human polyvalent immunoglobulin serum and with anti-human κ or λ-chain serum (DAKO) for 30 min at room temperature without adding sodium azide in the solution. After thorough washing with RPMI-1640 medium, they were tested to form SEn rosettes, incubating for 15 min at 37°C (the condition of "hot" rosettes). The reaction mixture was then left for 6 hr at room temperature to observe changes in the number of rosettes. (8) The KT cells were treated with trypsin (150 μg/ml, Merck) and DNase (10 μg/ml, Sigma) for 20 min at 37°C. The number of SEn rosettes and SIg-bearing cells were counted before and after the treatment. The treated KT cells were then cultured for 3 days in RPMI-1640 medium containing 20% fetal calf serum. (9) Trials to separate the rosette-forming cells from the rosette-nonforming cells were also performed. For this purpose, the KT cells or normal PBL that were reacted once with SEn were placed on a Percoll solution (specific gravity of the solution was adjusted to 1.077, Pharmacia Fine Chemicals) and centrifuged at 1500 g.

Immunochemical Studies

Rabbit antisera against human μ, κ, and λ chains previously absorbed with untreated mouse sera were used with Ouchterlony's method to detect human immunoglobulins in the tumor homogenates or in plasma of nude mice bearing walnut-sized tumors. A peroxidase–antiperoxidase (PAP) method was applied to demonstrate human μ, κ, or λ chains in trypsin-treated paraffin sections of the xenotransplanted tumors.

RESULTS

Transplantation to Nude Mice

A part of the fresh biopsy specimen was transplanted into the subcutis of two preirradiated nude mice. About 7 wk later, walnut-sized tumors were formed in both mice. Since then, serial transplantations to preirradiated nude mice have been carried out every 1.5 mo. Figure 1 shows a tumor-bearing mouse in the 19th passage.

Histologic Studies

The neck lymph node taken at biopsy showed the histologic picture of malignant lymphoma of the diffuse undifferentiated non-Burkitt type (Rappaport) (Fig. 2). The xenotransplanted tumors were histologically similar to the biopsy material except for an indistinct starry-sky appearance.

Surface Marker Studies

The surface markers on the KT tumor cells, studied on the initial biopsy material and the xenotransplanted tumors in the third, sixth, and seventh passages (P3, P6, P7), are summarized in Table 1. Receptors for SEn were always positive in 34%–54% of the KT tumor cells during the passages, while 20%–53% of the cells also manifested SIg (μ) on their surfaces. A monoclonal surface κ-chain was identified on the P3 tumor cells. SIg μ was also detected when the F(ab′)2 fragments of IgG of the rabbit antiserum were used instead of the whole IgG. In addition, 71%–78% of the KT cells constantly possessed the receptors for the Fc portion of IgG but not of IgM. The receptors for C3b or C3d, detected by EACm or EACm, respectively, were positive on the P3 cells, but were negative on the P6 and P7 cells. The cytotoxicity tests using anti-T or anti-"Ia" sera revealed the presence of the Ia-like antigens on the cells, while T-cell antigens failed to be proven. The immunofluorescence study also revealed Ia-like antigens on most of the KT cells.

Evaluation of SEn Rosettes

None of the KT cells formed rosettes with sheep erythrocytes (SE) when the neuraminidase treatment was omitted (Table 2). Therefore, we examined a temperature dependency of the rosettes. A comparable number of the rosettes (18%–20%) were observed, even though the incubation was undertaken only for 15 min at 4°C, 20°C, or 37°C. The AET treatment of SE had no effect on the rosette formation (Table 2). On the other hand, the AET treatment of the KT cells paradoxically enhanced the rosette formation (from 16% to 38%), while the normal PBL treated with AET failed to form SEn rosettes.

To rule out the interaction between neuraminidase on SEn and "neuraminidase-receptors" on the KT cells, the rosette formation tests using enzyme-treated erythrocytes from other species, such as man (AB type), horse, mouse, and rabbit, were undertaken. In any case,
rosette-forming cells were hardly found, while the percentage of SEn rosettes was 25% in this experiment. In addition, a significant number of the enzyme-treated KT cells (16%) could also form SEn rosettes. SE treated with neuraminidase plus EDTA reacted to the KT cells only in a small number (Table 2).

In order to discover the significance of monoclonal SIg in the rosette formation with SEn, blocking tests using antipolyvalent immunoglobulin or anti-light-chain antisera were performed under the conditions of "hot" rosettes (Table 3). Antipolyvalent immunoglobulin or anti-\(\kappa\)-chain serum almost completely abolished the SEn rosettes, in sharp contrast to anti-\(\lambda\)-chain serum. After the mixtures were left for 6 hr at room temperature, the rosette-forming capacity was significantly restored, probably due to the capping phenomenon followed by a de novo synthesis of the monoclonal SIg by the KT cells. With trypsinization, SEn rosettes on the KT cells decreased to about one-third of the control, while SIg almost completely disappeared. During the following 3-day culture period, SIg was partially reexpressed, but SEn rosette formation was not significantly recovered. It is likely that this discrepancy resulted from a decreased viability of the KT cells, which fell to 45% after 48 hr of the culture.

Several trials to separate the rosette-forming KT cells from the rosette-nonforming cells did not succeed, although the normal PBL forming SEn rosettes were easily collected at the bottom of the centrifugation tubes with more than 95% purity. It was highly probable that the rosettes between the KT cells and SEn were loosely and weakly formed in comparison with the rosettes between the normal PBL and SEn.

### Table 1. Surface Markers on KT Cells

<table>
<thead>
<tr>
<th></th>
<th>B-LN* (%)</th>
<th>(P_t) (%)</th>
<th>(P_d) (%)</th>
<th>(P_r) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>42</td>
<td>49</td>
<td>34</td>
<td>54</td>
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<tr>
<td>OxEach</td>
<td>5</td>
<td>45</td>
<td>0</td>
<td>0</td>
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<td>OxEACm</td>
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<tr>
<td>OxEAlgG</td>
<td>71</td>
<td>78</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>OxEAlgM</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slg(\kappa)</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slg(\lambda)</td>
<td>29</td>
<td>20</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Slg(\gamma)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slg(\delta)</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slk</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTLAt</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>&quot;(Ia^d)&quot;-like Ag</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Biopsy lymph node.
†Human T lymphocyte antigen(s).

### Table 2. Rosette Formation Tests of KT Cells or Normal PBL Using SE With or Without Treatments Under the Standard Rosetting Condition

<table>
<thead>
<tr>
<th></th>
<th>KT Cells (%)</th>
<th>Normal PBL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>SEn</td>
<td>19</td>
<td>70</td>
</tr>
<tr>
<td>SE (n + EDTA)</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td>SE (AET)</td>
<td>0</td>
<td>74</td>
</tr>
</tbody>
</table>
**Table 3. Effects of Antisera on SEn Rosettes With KT Cells Under the Condition of “Hot” Rosettes**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>∆7°C 15 min Only (%)</th>
<th>After Incubation for 6 hr at RT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Antipolyvalent serum</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Anti-kappa serum</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Anti-lambda serum</td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

**Immunoochemical Studies**

In homogenates of the P13 xenotransplanted tumor, human μ-chain was demonstrated in Ouchterlony’s agar plates, while it was undetectable in the mouse sera. The immunohistochemical study with the PAP method revealed a few tumor cells containing human μ and κ chain in the cytoplasm.

**DISCUSSION**

Lymphocytes with “dual markers” have been reported in normal individuals and in patients with chronic lymphocytic leukemia, lymphosarcoma cell leukemia, hairy cell leukemia, and malignant lymphoma. Cultured lymphoid cell lines with dual markers have also been reported. In these reports, however, C3 receptors have often been used as a marker of B cells. Since some normal cortical thymocytes and a few normal PBL may possess the complement receptors as well as the SE receptors, it is reasonable to assume that normal or neoplastic lymphocytes having both SE and complement receptors without SIg consist of a subpopulation of T cells. Moreover, it has been reported that a set of antigens are common to some leukemic B cells of chronic lymphocytic leukemia and T-cell lineages.

The present case is a peculiar lymphoma composed of cells that express both monoclonal SIg (μ, κ) and “receptors” for SE. Surface markers of the tumor cells (KT) remained unchanged during the serial transplantations to nude mice, except for the disappearance of the C3 receptors. The KT cells carried constant Fcγ receptors. They expressed “Ia-like” antigens, but the anti-T-serum failed to demonstrate the T-cell antigens on their surfaces. The immunoochemical study on the xenotransplanted tumor revealed the presence of μ or κ-chain immunoreactivities within the KT cells. Since all data except SE rosettes were compatible with B-cell natures, the SE rosettes formed by the KT cells needed to be reevaluated. The detailed reevaluation showed peculiar properties of the SE rosettes on the KT cells, as summarized in the following: (1) The KT cells did not form rosettes with neuraminidase-untreated or AET-treated SE. (2) SE rosettes on the KT cells did not show a temperature dependency. (3) Neuraminidase-treated erythrocytes from other species such as man (AB type), horse, mouse, and rabbit could not be substituted for the rosette formation, and the neuraminidase treatment on the KT cells did not change their rosette-forming capacity. (4) Preincubation of the KT cells with antipolyvalent immunoglobulin or anti-κ-chain serum abolished the SE rosette formation, and the rosette-forming capacity of the KT cells was restored after leaving the reaction mixture for 6 hr at room temperature. (5) AET treatment of the KT cells showed an enhanced reactivity to SE, while the same treatment of normal PBL completely abolished the SE rosette formation. (6) Trypsinization of the KT cells resulted in the decrease of the SE rosettes and in the disappearance of the SIg. (7) SE rosettes on the KT cells were too loose to be separated from nonrosetting cells by the Percoll gradient centrifugation method.

It is concluded that SE rosettes formed by the KT cells are greatly different from those formed by the classical SE receptors and that they were mediated through the monoclonal SIg, which can specifically bind sheep erythrocyte antigen(s) exposed after neuraminidase treatment. It is also likely from the blocking experiment with antiimmunoglobulin sera that the SE binding capacity was newly synthesized by the KT cells, although this could not be demonstrated in the trypsinization experiment. The interaction between neuraminidase on SE and neuraminidase receptors on the KT cells was disregarded because of the results of the experiments using either neuraminidase-treated erythrocytes from other species or the enzyme-treated KT cells.

Neoplastic lymphocytes simultaneously showing both monoclonal SIg and the classical SE rosettes occur very rarely, and the normal lymphocyte population bearing such truly dual markers has not yet been described in man. Leukemia or lymphoma cells with both SIg and SE rosettes have been reported by Brouet and Halper. In both reports, the neoplastic cells synthesized the monoclonal SIg, which was responsible for the rosettes since specific antimmunoglobulin sera successfully inhibited the rosetting. Brouet’s case showed SIg with an anti-Forssman antigen activity. One of three cases reported by Halper was accompanied by a monoclonal serum cryoglobulin that also reacted to human erythrocytes. It is of interest that the KT cells specifically reacted only with SE, but not with SE or enzyme-treated erythrocytes of other species. This fact excludes the possibility that the monoclonal SIg on the KT cells shows a Forssman antibody activity or a nonspecific cold agglutinin reactivity. (It is well known that Forssman antigen is
positive on sheep, horse, and human AB type erythrocytes, but is negative on mouse and rabbit erythrocytes.

In order to classify lymphoproliferative disorders properly, combination studies of a large panel of surface markers would be needed, and the meaning of each marker study must be judged with careful evaluations. In the present case, detailed analyses of the SEN rosette formation revealed the expression of another new receptor on neoplastic lymphocytes.

REFERENCES

5. Mendes NF, Tolnai MEA, Silveira NPA, Gilbertson RB, Metzgar RS: Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. J Immunol 111:860–867, 1973
A B-lymphoma cell line that forms rosettes with neuraminidase-treated sheep erythrocytes through monoclonal surface immunoglobulin

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