Humoral Helper Activity for B-Cell Differentiation Released From Non-Hodgkin’s Lymphoma Cells Having Both SRBC and Complement Receptors in the Pokeweed Mitogen System

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The majority of lymphoid cells from a patient with non-Hodgkin’s lymphoma with leukemic transformation were demonstrated to carry receptors for both sheep erythrocytes and complements by the combined rosette assay using neuraminidase-treated sheep erythrocytes and complement-coated zymosan beads. Most of them were considered morphologically lymphoblasts and were positive for acid phosphatase staining. Terminal deoxynucleotidyl transferase activity was not detected in these cells. Lymphoid cells from this patient did not respond to the stimulation with phytohemagglutinin-P, concanavalin-A, and pokeweed mitogen (PWM). When these cells were cultured with PWM for 7 days, no plasma cell was generated. Although only few plasma cells were generated in the PWM-stimulated culture of normal purified B cells alone, the addition of the patient’s cells to purified normal B cells resulted in a markedly enhanced generation of plasma cells in response to PWM, as was the case with normal T cells. But leukemic cells either from a patient with T-cell leukemia not having complement receptors or from a patient with null-cell leukemia showed no enhancing ability in B-cell differentiation. In addition, the culture supernates of the patient’s cells obtained after 24-hr PWM stimulation had an ability to promote B-cell differentiation comparable in activity to those from the PWM-stimulated normal T cells.

A small proportion of peripheral blood lymphocytes in healthy subjects have been shown to have receptors for both sheep erythrocytes (SRBC) and complements.1,2 Stein and Müller-Hermelink3 reported that in human fetal thymuses, relatively high percentage of thymocytes (20%-30%) bear complement receptors and relative proportion of these cells in thymus declines with advancing gestation week. Gupta et al.4 reported that in a case of a thymectomized infant, his circulating T cells bore a high percentage (about 25%) of complement receptors even after 10 mo of thymectomy. These data may suggest that T cells with complement receptors are situated in one stage of T-cell development, but it remains unclear that these doubly marked lymphocytes are a functional subset of T lymphocytes.

In the lymphoproliferative diseases, it may be reasonable to speculate that neoplastic cells are driven from the clonal proliferation of lymphocytes at various maturation stages. Neoplastic cells from some T-cell malignancies have been shown to have the regulatory functions on the differentiation of normal B cells.5-7 Thus, the functional analyses of the neoplastic cells may also allow the supposition of developmental functions of normal lymphocytes in the immune system. In many lymphoid malignancies, it has been reported that various proportions of lymphoid cells had rosetting ability with SRBC and complement-coated indicators.8-10 Chiao et al.11 recently reported the functional analysis of leukemic cells that possessed receptors for both SRBC and complements. They suggested that the doubly marked leukemic cells enhance adult B-cell differentiation in the plaque-forming assay.

In this study, we report a case of non-Hodgkin’s lymphoma with leukemic transformation. On some occasions, about 90% of the neoplastic cells formed double rosettes of SRBC and complement-coated zymosan (Zy) beads. We examined the helper function of these cells for B-cell differentiation by coculturing them with normal purified B cells in the presence of pokeweed mitogen (PWM) or by adding the culture supernates from these cells incubated with PWM to normal B cells.

**MATERIALS AND METHODS**

**Separation of Lymphoid Cells**

Normal peripheral blood lymphocytes were obtained from healthy male volunteers 24-32 y old. Lymphoid cells from a patient with non-Hodgkin’s lymphoma with leukemic transformation were obtained at various times of clinical course. Leukemic cells from other two patients with ALL, T-cell ALL, and null-cell ALL were obtained and used as neoplastic controls. Cells were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation.12 Bone marrow mononuclear cells were separated from heparinized bone marrow aspirations diluted with a large volume of phosphate-buffered saline (PBS, pH 7.4) by Ficoll-Hypaque gradient. Mononuclear cells in cerebrospinal fluid (CSF) were obtained on an occasion of central nervous system (CNS) relapse of leukemic lymphoma.

**Preparation of Normal T and B Cells**

Normal T cells were separated from peripheral blood lymphocytes by rosette formation with neuraminidase-treated SRBC13 through Ficoll-Hypaque gradient. Rosetted T cells were obtained...
LYMPHOMA WITH HELPER T CELL CHARACTER

1075

from the pellet of the gradient. Attached SRBC were lysed by adding 0.01 M Tris-0.84% NH₄Cl solution. Obtained T-cell populations were constituted of 95% or more SRBC rosette-forming cells and of less than 1% surface immunoglobulin (Ig) bearing cells. Normal B cells were obtained from the gradient interface by the following method. Cells in the gradient interface were washed twice in PBS and layered on the tissue culture dishes (no. 3002, Falcon) at a cell concentration of 10/3/3 ml medium/dish with 20% heat-inactivated fetal bovine serum (FBS, Gibco). The dishes were incubated for 90 min at 37°C in a 5% CO₂ incubator. Nonadherent cells were harvested, resuspended with SRBC, and layered on the second Ficoll-Hypaque gradient. Two-step depletion of SRBC rosette-forming cells yielded less than 0.5% contamination of T cells in normal B-cell populations. These purified B cells contained about 10% monocytes detected by nonspecific esterase staining.14

Surface Marker Determinations

T lymphocytes were determined by the spontaneous rosette formation with neuraminidase-treated SRBC as described elsewhere.15 Surface Ig-bearing cells were detected by direct immunofluorescence technique using fluorescein isothiocyanate (FITC) conjugated goat anti-human Ig antibody (IgG + IgM + IgA, Behringwerke AG, Marburg, West Germany).16 Cells with Fc receptor for IgG were determined by rosette formation with ox erythrocytes coated by IgG fraction of rabbit anti-ox erythrocyte antibody as described previously.17 Complement receptors were detected by two methods using SRBC or zymosan beads as indicators. SRBC were incubated with rabbit anti-SRBC IgG antibody for 30 min at 37°C and then sensitized with fresh human serum (1:50 dilution) for 15 min at 37°C (EAC).18 Twenty-five microliters of 5 x 10⁵/ml EAC and 25 μl of cell suspension (10⁷/ml) were mixed and incubated at 37°C for 30 min without shaking. Then the mixture was gently resuspended and cells binding three or more EAC were considered EAC rosette-forming cells. Zymosan beads were used as another indicator for complement receptors. Zymosan beads (Zy, Sigma Chemical Company, St. Louis, Mo.) were coated with complement as described by Huber and Wigzell18 with minor modifications. Briefly, Zy were swollen in boiling water for 30 min, washed in gelatin-veronal buffer (GVB') containing 0.03 M CaCl₂ and 0.1 M MgCl₂, and resuspended in GVB' at a concentration of 1 mg/ml. Then, Zy were incubated with fresh human serum (0.5 ml serum for 1 mg Zy) for 30 min at 37°C, washed 3 times, and resuspended in GVB' at a final concentration of 0.5 mg/ml (Zy-C). Twenty-five microliters of cell suspension (10⁷/ml) and 25 μl of Zy-C were mixed and centrifuged at 100 g for 5 min. The pellet was incubated for a further 30 min at 4°C and gently resuspended. Cells binding two or more Zy-C were considered positive. Cells having both SRBC and complement receptors were detected as follows: 25 μl of cell suspension (10⁷/ml), 25 μl of neuraminidase-treated SRBC (2 x 10⁵/ml), 25 μl of heat-inactivated FBS that had been absorbed with SRBC and 25 μl of Zy-C were mixed and centrifuged at 100 g for 5 min. The pellet was kept at 4°C for 30 min and gently resuspended. Doubly marked cells bearing three or more SRBC and two or more Zy-C in 200 cells were counted.

Cellular Proliferative Responses to Mitogens

Cells (10⁶) suspended in 0.2 ml of RPMI 1640 medium (GIBCO) containing 10% heat-inactivated FBS were cultured in each well of tissue culture plates (no. 3042, Falcon). A quantity of 10 μg/ml phytohemagglutinin-P (PHA-P, Difco Lab., Detroit, Mich.), 10 μg/ml concanavalin-A (Con-A, Difco Lab.), and 5 μg/ml of pokeweed mitogen (GIBCO) were added to each well. PHA-P and Con-A cultures were incubated for 3 days and PWM cultures for 5 days at 37°C in a humidified atmosphere of 5% CO₂ in air. Then, 1 μCi/ml of ³H-thymidine (New England Nuclear, Boston, Mass.) was added to each well during last 24 hr of culture. Cells were collected with a semiautomatic multiple cell harvester (Labo Science, Osaka, Japan) and incorporation of ³H-thymidine into cells was measured by a liquid scintillation counter. The results were expressed as mean cpm ± 1 SD in triplicate cultures.

Culture Supernates

Graded numbers of normal T cells and 2 x 10⁶/ml of lymphoid cells of the patient obtained from bone marrow aspiration (October 4, 1979, double rosette 79%), from CSF (November 19, 1979, double rosette 91%), and from peripheral blood (April 8, 1980, double rosette 90%) were cultured in RPMI 1640 medium containing 20% heat-inactivated FBS with PWM (25 μg/ml) for 24 hr. Then, cells were spun down by centrifugation at 400 g for 10 min, and cell-free culture supernates were obtained and stored at -20°C until use. The culture supernates from 2 x 10⁶/ml of another two leukemic cells were also obtained as described above. The helper activity in the culture supernates for B-cell differentiation was assessed as described below.

Assay of Helper Activity for B-Cell Differentiation

Normal purified B cells were suspended in RPMI 1640 medium supplemented with 0.3 mg/gl l-glutamine, 200 U/ml penicillin, 10 μg/ml gentamicin, and 20% heat-inactivated FBS at a cell concentration of 10⁷/ml. In the coculture experiment, 10⁷ or 5 x 10⁷ normal T cells or the patient's cells obtained from peripheral blood on April 8, 1980 (double rosette 90%) were mixed with 0.5 ml of normal B cells at a final volume of 1 ml and mixtures were cultured with PWM (optimal dose, 25 μg/ml) at 37°C. After 7 days, cells were harvested and tested for their cytoplasmic Ig as described below. In another experiment, helper activity in the culture supernates was assessed. Of the culture supernates, 0.5 ml obtained from normal T cells, from the patient's cells, and from two leukemic cells was added to 0.5 ml of normal B-cell suspension. The mixtures were cultured at 37°C for 7 days without PWM. At the end of culture, cells containing cytoplasmic Ig were detected.

Detection of Cells Containing Cytoplasmic Immunoglobulins

Cytoplasmic Ig-producing cells were detected by direct immunofluorescence technique described elsewhere.20 Briefly, after 7 days of culture, cells were counted by hemocytometer chamber, washed twice in PBS, mounted on slide glass, air dried, fixed in 5% acetic acid-95% ethanol at -20°C for 20 min, and stained with FITC-conjugated goat anti-human Ig antibody (IgG + IgM + IgA, 1:20 dilution, Behringwerke) for 30 min at room temperature. Cytoplasmic Ig-producing cells (plasma cells) in 1000 cells were counted by using an Olympus fluorescence microscope. The absolute number of plasma cells was calculated by multiplying the percentage of plasma cells by the number of cells recovered from one tube.

CASE REPORT

A 9-yr-old Japanese girl was first admitted to the hospital because of left-sided massive pleural effusion on August 5, 1978. Physical examination revealed splenomegaly, 5 cm below the left costal margin, without any peripheral lymphadenopathy. A left-sided thoracentesis disclosed neoplastic cells. The bone marrow aspiration also revealed tumor cells, which constitute 76% of the total nucleated cells indistinguishable from ALL morphologically. These cells were uniform in appearance with round or slightly identified nuclei and scanty cytoplasm. They were negative with peroxidase reaction. Although there was no anemia in peripheral
blood, white blood cell count was 32,300/cu mm, with 54% neutrophils, 38% lymphocytes, 4% eosinophils, and 4% immature lymphoblastoid cells. The erythrocyte sedimentation rate was within normal range. The urinary spot test for vanillylmandelic acid was negative. The patient was diagnosed as having non-Hodgkin's lymphoma (diffuse lymphoblastic21) with leukemic transformation, although lymph node biopsy was not done. She was begun on a combined chemotherapy and radiotherapy program that involved the administration of LSA2L, (cyclophosphamide, vincristine, prednisone, methotrexate, and daunomycin)22'23 and local radiation to mediastinum. After 2 mo therapy, bone marrow picture attained to MI marrow (lymphoblast count was less than 5%) and chest X-P showed remarkable improvement of pleural effusion. A complete remission was sustained until October 3, 1979, when hematologic relapse occurred with cervical lymph node swellings. One month later, CNS involvement was also observed (November 19, 1979). Cell count in the cerebrospinal fluid was 2584/cu mm and 95% or more were lymphoblast as judged by stained morphology. On this time, white blood cell count was 20,600/cu mm, with 85% lymphoblast, 7% lymphocytes, and 8% neutrophils. But the bone marrow picture sustained MI marrow. Neoplastic cells from CSF were positive for acid phosphatase24 but were negative for nonspecific esterase staining. Terminal deoxynucleotidyl transferase (TdT) activity25 was not detected in these cells by indirect immunofluorescent assay kit (Bethesda Research Lab. Inc.). By treatment with COAP (cyclophosphamide, vincristine, cytocin arabinoside, and prednisone)26 and 1-asparaginase, a partial remission continued until April 18, 1980, when the second hematologic relapse was recognized. At this time, white blood cell count was 20,000/cu mm, with 94% lymphoblasts, 4% lymphocytes, and 2% neutrophils. Despite the aggressive treatment with antineoplastic drugs in combination with frequent platelets and granulocyte transfusions, gastrointestinal bleeding and pneumonia appeared and gradually progressed. She died from intracranial bleeding on June 30, 1980. Permission for autopsy was refused.

RESULTS

Surface Markers of the Patient's Cells

As shown in Table 1, a high proportion of lymphoid cells from this patient had rosetting ability with SRBC. On some occasions, 98% of cells formed rosette with SRBC (November 19, 1979 in CSF and April 18, 1980 in peripheral blood). At the same times, these cells formed rosettes with EAC and Zy-C. The percentages of SRBC rosette-forming cells were almost equal to the percentages of EAC or Zy-C rosette-forming cells. Double rosette technique using neuraminidase-treated SRBC and Zy-C revealed that both SRBC and complement receptors presented on the same cell simultaneously. Figure 1 shows the scanning electron micrograph of the doubly marked malignant cells in this patient. An elliptical Zy bead and deformed SRBC attached on the surface of the cell. Lymphoid cells obtained from CSF (November 19, 1979) and from peripheral blood (April 18, 1980) constituted more than 90% doubly marked cells. Surface Ig and Fc receptor for IgG were not detected on their surface. Cells from T-cell ALL constituted

<table>
<thead>
<tr>
<th>Date</th>
<th>Source</th>
<th>Neoplastic* Cells (%)</th>
<th>SRBC Rosette</th>
<th>Surface Ig</th>
<th>EA-IgG Rosette</th>
<th>EAC Rosette</th>
<th>Zy-C† Rosette</th>
<th>SRBC † Zy-C</th>
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<tbody>
<tr>
<td>October 4, 1979</td>
<td>BM †</td>
<td>92</td>
<td>82</td>
<td>3</td>
<td>2</td>
<td>85</td>
<td>87</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>PB †</td>
<td>ND †</td>
<td>85</td>
<td>2</td>
<td>4</td>
<td>78</td>
<td>80</td>
<td>71</td>
</tr>
<tr>
<td>October 30</td>
<td>BM</td>
<td>87</td>
<td>40</td>
<td>ND</td>
<td>11</td>
<td>ND</td>
<td>57</td>
<td>37</td>
</tr>
<tr>
<td>November 19</td>
<td>PB</td>
<td>85</td>
<td>77</td>
<td>ND</td>
<td>9</td>
<td>82</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>95</td>
<td>98</td>
<td>ND</td>
<td>0</td>
<td>98</td>
<td>93</td>
<td>91</td>
</tr>
<tr>
<td>April 18, 1980</td>
<td>PB</td>
<td>94</td>
<td>98</td>
<td>ND</td>
<td>0</td>
<td>97</td>
<td>92</td>
<td>90</td>
</tr>
<tr>
<td>Normal Control</td>
<td>PB</td>
<td>(range)</td>
<td>(59–78)</td>
<td>(5–15)</td>
<td>(22–38)</td>
<td>(15–34)</td>
<td>(12–29)</td>
<td>(0.5–4.0)</td>
</tr>
</tbody>
</table>

*Neoplastic cells were judged by stained morphology.
†Zy-C, complement-coated zymosan beads; BM, bone marrow; PB, peripheral blood; ND, not done; CSF, cerebrospinal fluid.
LYMPHOMA WITH HELPER T CELL CHARACTER

Table 2. Proliferative Responses to Mitogens

<table>
<thead>
<tr>
<th>Mitogens</th>
<th>BM†</th>
<th>CSF‡</th>
<th>PB§</th>
<th>Control (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-P</td>
<td>353 ± 46§</td>
<td>130 ± 17</td>
<td>11,229 ± 735</td>
<td>43,022 ± 4,602</td>
</tr>
<tr>
<td>Con-A</td>
<td>260 ± 30</td>
<td>184 ± 11</td>
<td>5,376 ± 889</td>
<td>39,019 ± 1,563</td>
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<tr>
<td>None</td>
<td>368 ± 35</td>
<td>122 ± 20</td>
<td>3,483 ± 205</td>
<td>416 ± 40</td>
</tr>
<tr>
<td>PWM</td>
<td>201 ± 54</td>
<td>279 ± 79</td>
<td>6,009 ± 560</td>
<td>21,104 ± 4,291</td>
</tr>
<tr>
<td>None</td>
<td>164 ± 65</td>
<td>267 ± 80</td>
<td>3,369 ± 46</td>
<td>305 ± 162</td>
</tr>
</tbody>
</table>

*Cells from a patient with non-Hodgkin’s lymphoma having both SRBC and complement receptors.
†BM, bone marrow obtained on October 4, 1979.
‡CSF, cerebrospinal fluid obtained on November 19, 1979.
§PB, peripheral obtained on November 19, 1979.
¶³H-thymidine uptake, mean count ± 1 SD/min/10⁶ cells.

more than 90% lymphoblasts as judged by stained morphology, and 98% of them formed rosette with SRBC. Other surface markers were not detected on their surface (data not shown). Cells from null-cell ALL consisted of 96% lymphoblast and none of them had any surface markers examined in this study (data not shown).

Cellular Proliferative Responses to Mitogens

Lymphoid cells from various sources of this patient were stimulated with PHA-P, Con-A, and PWM. Cells from bone marrow (October 4, 1979) constituted 92% lymphoblast and 79% double rosette-forming cells. Cells from peripheral blood (November 19, 1979) constituted 85% lymphoblast and 73% double rosette-forming cells. Cells from CSF (November 19, 1979) constituted 95% lymphoblast and 91% double rosette-forming cells. As shown in Table 2, lymphoid cells from bone marrow aspiration or from CSF that constituted a high proportion of lymphoblast and doubly marked cells, did not respond to any mitogens. But cells from peripheral blood poorly responded to these mitogens. These responses might be due to contaminated normal T or B cells.

Helper Activity of the Patient’s Cells for B-Cell Differentiation

Normal purified B cells (5 x 10⁵ cells) were cocultured with 10⁵ or 5 x 10⁵ patient’s cells obtained from peripheral blood on April 18, 1980 or normal T cells in the presence of PWM. The results are shown in Fig. 2. The patient’s cells showed a significant helper activity on the differentiation of normal B cells comparable to those of normal T cells. When purified normal B cells were cultured solely with PWM, a negligible number of plasma cells were generated. In the culture of the patient’s cells alone with PWM, no plasma cell was detected (data not shown).

Helper Activity in the Culture Supernates of the Patient’s Cells

It is well known that normal T cells can promote B-cell differentiation through humoral factors secreted in the culture fluid of T cells stimulated with PWM. In Fig. 3, helper activities in the culture supernates from various numbers of autologous or allogeneic normal T cells were shown. The number of plasma cells increased according to the increment of
patient's cells and two other leukemic cells. The results were shown in Figure 4. All of the culture supernates of the patient's cells obtained from bone marrow (October 4, 1979), from CSF (November 19, 1979), and from peripheral blood (April 18, 1980) showed significant helper activities for B-cell differentiation. Their helper activities were comparable to those from normal T cells. But the culture supernates of cells from T-cell ALL and null-cell ALL patients did not show any helper activity. The culture supernate of the patient's cells incubated for 24 hr without PWM could not promote B-cell differentiation (data not shown).

DISCUSSION

In this study, we presented a case of non-Hodgkin's lymphoma with leukemic transformation. Lymphoid cells from various sources of this patient constituted more than 90% doubly marked cells having both SRBC and complement receptors. These cells were considered lymphoblast as judged by stained morphology. Chiao et al. previously reported a case of ALL whose leukemic cells had both SRBC and complement receptors. They showed that the doubly marked leukemic cells enhanced the generation of plaque-forming cells in the coculture with normal adult peripheral blood lymphocytes. Their leukemic cells did not respond to PHA and Con-A but poorly responded to PWM. In our case, malignant cells with double marker did not respond to these mitogens. We examined the helper activity for in vitro B-cell differentiation by coculturing the patient's cells with normal purified B cells in the presence of PWM. When the patient's cells were cultured with PWM alone, no plasma cell was generated. But, in the coculture

![Fig. 3. Helper activity in the culture supernates from graded numbers of autologous or allogeneic normal T cells incubated with pokeweed mitogen for 24 hr. The culture supernates were added to normal purified B-cell suspension at a final concentration of 50%, and these mixtures were incubated without PWM. The results were shown as the absolute number of plasma cells generated in each tube. Vertical bars represent ±1 SD from the mean of three experiments.](image)

![Fig. 4. Helper activity in the culture supernates from the patient's cells (2 × 10⁶/ml) incubated with pokeweed mitogen for 24 hr. The culture supernates were added to normal purified B-cell suspension at a final concentration of 50% and cultured without pokeweed mitogen. The results were shown as the absolute number of plasma cells generated in each tube. Vertical bars represent ±1 SD from the mean of five experiments of normal T cells. BM, bone marrow lymphoid cells (October 4, 1979); CSF, cells from cerebrospinal fluid (November 19, 1979); PB, peripheral blood lymphoid cells (April 8, 1980).](image)
system, these cells showed a significant helper activity on the differentiation of normal B cells. In the culture of normal purified B cells alone with PWM, a negligible number of plasma cells were generated. Therefore, we assume that the marked generation of plasma cells in the coculture resulted from the helper function of the patient's cells, not from those of normal T cells contaminated in B-cell population.

It is well known that normal purified B cells can differentiate into plasma cells in the presence of culture supernates obtained from normal T cells stimulated with PWM for 24 hr. According to this evidence, we examined whether the neoplastic cells from this patient can promote the differentiation of normal B cells through humoral factors secreted in the culture after 24-hr PWM stimulation. The culture supernates from the patient's cells as well as those from normal T cells were able to provide sufficient helper activities for B-cell differentiation (Fig. 4). But the culture supernates of leukemic cells from T-cell ALL not having complement receptors and from null-cell ALL did not show any helper activity. These results showed that the doubly marked neoplastic cells from this patient could promote B-cell differentiation through humoral helper factors secreted within 24 hr of the PWM stimulation. Even if a small number of normal T cells contaminated the patient's cells, the number of normal T cells might not be sufficient to exert such helper activities observed in the culture supernates of the patient's cells.

Recently, Semenzato et al. reported the doubly marked leukemic cells with cytotoxic activities in ALL patient. These leukemic cells poorly responded to PHA and Con-A stimulation but showed high activities in spontaneous cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity. In our study, we did not examine these cytotoxic activities in the patient's cells. Therefore, the possibility that the doubly marked neoplastic cells with both SRBC and complement receptors have two different functions was not elucidated.

Recent investigations and our results showed that highly pure doubly marked cells in the neoplastic diseases exert some functions (helper or cytotoxic) on normal lymphocytes in vitro. But the functions of normal T cells with complement receptors are still unclear.

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