Heterologous Antiserum to Chemically Induced Rat Non-T, Non-B Leukemia and Its Application to Characterization of Rat Leukemias

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ABSTRACT

Antisera to the 1-butyl-1-nitrosourea-induced "non-T, non-B" rat leukemia line DBLA-6 were raised in rabbits. Following absorption with syngeneic hepatoma cells, the antisera were very similar in specificity to antisera raised to rat Thy-1 antigen. Anti-DBLA-6 serum was cytotoxic in the presence of complement against 70 to 90% of thymocytes and 40 to 50% of neonatal spleen cells. In contrast, no significant cytotoxicity was observed against cells from bone marrow, lymph node, spleen, and peritoneum. An absorption test revealed that an antigen recognized by anti-DBLA-6 serum was present in brain tissue but absent in liver and kidney tissues. Nineteen rat leukemias and lymphomas were divided into six groups based on antigenic and morphological characteristics and the presence of receptor for guinea pig red blood cells. These tumors were investigated for the presence of the antigen recognized by anti-DBLA-6 serum. Of the leukemias and lymphomas studied, anti-DBLA-6 serum reacted with all thymic (Group 1) and extrathymic (Group 2) lymphomas and unclassified leukemias (Groups 3 and 4), while all myelogenous leukemias (Group 5) and erythroleukemias (Group 6) were negative. The position of leukemias and lymphomas reactive with anti-DBLA-6 serum in the lymphocyte maturational pathway is discussed.

INTRODUCTION

Induction of different types of leukemias and lymphomas in rats by various chemicals and murine leukemia viruses has been reported by many investigators (6, 8, 13, 15-17). For many years, the classification of rat leukemias and lymphomas has been based essentially on morphological and cytotoxic criteria. Recently, several cell surface markers of lymphocytes and other hematopoietic cells have been reported in rats (3, 7, 12, 21). However, cell surface markers are not yet routinely used for the characterization of rat leukemia and lymphoma, although several murine leukemias and lymphomas have been well characterized by cell surface markers (20).

We have previously reported the serological characterization of the BNU3-induced rat leukemia lines, DBLA-1, -6, and -9, and its Application to Characterization of Rat Leukemias

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3 The abbreviations used are: BNU, 1-butyl-1-nitrosourea; GPRBC, guinea pig red blood cells; RF, rosette formation; PBS, phosphate-buffered saline (NaCl, 0.145 g; Na2HPO4, 0.725 g; KH2PO4, 0.212 g; water, 1000 ml); ALS, antilymphocyte serum; CI, cytotoxicity index; RSTA, rat-specific thymus antigen; BMLA, bone marrow lymphocyte antigen.

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**Procedure for RF.** The method described by Takeichi et al. (22) was used. RBC were obtained from guinea pigs by cardiac puncture with heparin as the anticoagulant. The GPRBC were washed twice in PBS and concentrated to $5 \times 10^7$ cells/ml in PBS and 10% fetal bovine serum. Lymphoid or tumor cell suspension (0.1 ml; $5 \times 10^6$ cells/ml) was mixed with 0.1 ml of the GPRBC suspension and incubated for 5 min at 37°. The mixture was centrifuged at 1500 rpm for 5 min. Approximately 0.1 ml of the supernatant was removed by aspiration with a capillary pipet, and the layer cell pellet was gently resuspended by shaking. One drop of the cell suspension was mounted onto a glass slide and covered with a coverslip. Over 200 cells were counted, and all cells which had 3 or more RBC bound were considered positive.

**Membrane Immunofluorescence Test.** Five million normal lymphoid or tumor cells were incubated with 0.1 ml of 1:5 diluted goat anti-rat IgG serum conjugated with fluorescein isothiocyanate (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) for 30 min at 37°. The cells were then washed 3 times with PBS. The pellet was resuspended in 1 drop of 50% glycerol in PBS. Over 200 cells were counted, and all cells which displayed more than 2 sharply stained spots were considered positive.

**Antiserum Preparation.** Antisera against DBLA-6 cells were obtained by immunization of rabbits with $4 \times 10^6$ DBLA-6 cells 3 times i.v. at 2-week intervals and by bleeding them 1 week after the last injection. The antisera were absorbed 3 times with a 1:1 packed volume of syngeneic AH-66 hepatoma cells. ALS was produced by giving rabbits injections of WKA/Hok lymph node cells ($4 \times 10^6$ cells) 3 times i.v. at 2-week intervals, followed by bleeding 1 week after the last injection. The anti-serum was absorbed twice with a 1:1 packed volume of syngeneic KMT-17 fibrosarcoma cells and once with a 1:1 packed volume of WKA/Hok brain homogenate to absorb contaminating anti-Thy-1 antibodies. All antisera used were heat inactivated at 56° for 30 min and stored until used.

**Cytotoxicity Test.** Equal volumes (0.05 ml) of antiserum at doubling dilutions, a cell suspension ($5 \times 10^6$/ml) in MEM, and guinea pig complement were incubated at 37° for 45 min and washed in cold MEM. The proportion of dead cells was determined by microscopy with trypan blue solution. In each specimen, more than 200 cells were counted. The CI was calculated as

$$CI = \frac{c - t}{c}$$

where c is the percentage of unstained cells in the control sample and t is the percentage of unstained cells in the test sample. The mean control toxicity (target cells plus complement) for all tests was less than 10%. A CI higher than 0.20 was regarded as a positive reaction.

**Absorption Test.** Antiserum (0.1 ml), diluted 1 or 2 tubes below the CI of 0.5 to the target cells, was incubated with 0.1 ml of packed and washed cells or tissue homogenate at 4° for 60 min. The absorbed antisera were recovered after centrifugation (3000 rpm for 10 min) and tested for their residual cytotoxic activity against WKA/Hok thymocytes.

**Quantitative Absorption Test.** The capacity of different lymphoid cells to absorb the cytotoxic activity of antisera was determined by incubating 0.1 ml of adequately diluted antisera with decreasing numbers of cells from various sources. The test tubes were left at 4° for 60 min with occasional mixing. After centrifugation, the supernatants were tested for residual cytotoxic activity against thymocytes. The number of cells required to reduce the cytotoxicity to 50% was termed B and was expressed in relation to the number of target cells (A) required for the same reduction: absorbing capacity = $A/B \times 100$.

**RESULTS**

**Cytotoxic Activity of Rabbit Anti-DBLA-6 Serum on Various Normal Cells.** Since the 3 lines of BNU-induced rat leukemia, DBLA-1, DBLA-6, and DBLA-9, have almost the same morphological and cytochemical characteristics and cell surface differentiation antigens (10, 14), the DBLA-6 cell line was selected and antisera against DBLA-6 cells were produced in rabbits. Unabsorbed antiserum against DBLA-6 cells was strongly cytotoxic for various normal cells from a WKA/Hok rat (Chart 1). When the antiserum was absorbed 3 times with a 1:1 packed volume of syngeneic AH-66 hepatoma cells, it was still strongly cytotoxic for thymocytes and neonatal spleen cells but no longer cytotoxic for lymph node cells, spleen cells, bone marrow cells, peripheral blood lymphocytes, and peritoneal cells (Chart 2). This antiserum (anti-DBLA-6 serum) killed only 70 to 90% of thymocytes and 40 to 50% of neonatal spleen cells at high concentrations of the antiserum.

**Tissue Representation of Antigen Identified by Anti-DBLA-6 Serum.** Tissue representation of the antigen which is identified by anti-DBLA-6 serum was investigated by absorption tests. A 1:16 dilution of anti-DBLA-6 serum was absorbed with various types of normal lymphoid cells or tissue homogenates and then tested for the residual cytotoxicity against thymocytes. The calculated absorptive capacities were: thymocytes, 100; bone marrow cells, 4; and spleen cells, 1.7. Erythrocytes and lymph node cells failed to remove any cytotoxic activity, even at the highest cell density used (Chart 3). The anti-DBLA-6 serum was also absorbed with a 1:1 packed volume of brain, liver, and kidney homogenates (Table 1). The cytotoxic antibodies against thymocytes were completely absorbed by brain homogenate.
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J and WKA/Hok. When absorbed with AKR/J brain, the antiserum lost its cytotoxic activity on both BALB/c and AKR/J thymocytes but still reacted with thymocytes from a WKA/Hok rat. WKA/Hok brain completely absorbed the cytotoxic activity against all samples of target cells. These results indicate that antigens detected by anti-DBLA-6 serum consist of at least 3 sets of antigen, i.e., species-specific antigen, an antigen shared by the rat and AKR/J mouse, and an antigen cross-reacting between rat, mouse, and human.

Application of Anti-DBLA-6 Serum to Classification of Rat Leukemia. To investigate the potential of the anti-DBLA-6 serum as an antigen marker, the reactivity of anti-DBLA-6 serum with rat leukemias and lymphomas was examined.

Nineteen rat leukemias and lymphomas were studied with 3 kinds of cell marker; i.e., ALS as a marker of cells of lymphoid origin, anti-rat IgG serum as a marker of B-lymphocytes, and RF with GPRBC as a marker of thymus-derived cells. As shown in Table 3, the leukemias and lymphomas studied could be divided into 6 groups by combination with morphological analysis in May-Giemsa staining. Group 1, MuLV-induced thymic lymphomas; lymphoblastic, ALS (+), RF with GPRBC (+), and surface immunoglobulin (−). Group 2, MuLV-induced extrathymic lymphomas; lymphoblastic, ALS (+), RF with GPRBC (−), and surface immunoglobulin (−). Group 3, DBLA-1, DBLA-6, and DBLA-9; stem cell-like, ALS (+), RF with GPRBC (−), and surface immunoglobulin (−). Group 5, DBLA-10 and L1005; myelogenous granulocytic, ALS (−) and RF with GPRBC (−). Group 6, AL604, WFT-28E, and WFT-29E; erythroblastic, ALS (−), and RF with GPRBC (−).

Based on the results shown in Table 2, antiserum against homogenate, but liver and kidney homogenates had no absorbing capacities.

Species-specific and Cross-Reactive Antigens Detected by Anti-DBLA-6 Serum. It has been shown that heteroantisorbing capacities.

Chart 2. Cytotoxic activity of absorbed anti-DBLA-6 serum on normal WKA/Hok lymphoid cells. Upon thymocytes; O, lymph node cells; Δ, bone marrow cells; □, spleen cells; ■, neonatal spleen cells; □, peritoneal cells. Each point represents the mean of 3 to 10 experiments. Bars, S.E.

Chart 3. Quantitative absorption of anti-DBLA-6 serum with various normal WKA/Hok lymphoid cells. Upon thymocytes; O, lymph node cells; △, bone marrow cells; □, spleen cells; □, erythrocytes. Residual cytotoxicity of absorbed serum was tested against WKA/Hok thymocytes. Each point represents the average of duplicate samples from a representative experiment. Duplicate values agreed within 10%.

Table 1

Tissue representation of an antigen recognized by anti-DBLA-6 serum as determined by absorption test

<table>
<thead>
<tr>
<th>Absorbed with</th>
<th>Residual cytotoxicity*</th>
<th>Result of absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver homogenate</td>
<td>0.765</td>
<td>0.67</td>
</tr>
<tr>
<td>Kidney homogenate</td>
<td>0.74</td>
<td>0.58</td>
</tr>
<tr>
<td>Brain homogenate</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>DBLA-6 cells</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>0.75</td>
<td>0.68</td>
</tr>
<tr>
<td>None</td>
<td>0.86</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* Residual cytotoxicity of absorbed serum was tested against WKA/Hok thymocytes without further dilution (1:1) or diluted 1:2.

Average values of duplicate samples from a representative experiment. Duplicate values agreed within 5%.

Table 2

Residual cytotoxic activity of anti-DBLA-6 serum absorbed with brains from mouse, rat, and human

<table>
<thead>
<tr>
<th>Absorbed with brain from</th>
<th>BALB/c</th>
<th>AKR/J</th>
<th>WKA/Hok</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>0.005</td>
<td>0.86</td>
<td>0.90</td>
</tr>
<tr>
<td>AKR/J</td>
<td>0.00</td>
<td>0.03</td>
<td>0.83</td>
</tr>
<tr>
<td>WKA/Hok</td>
<td>0.01</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>Human</td>
<td>0.04</td>
<td>0.89</td>
<td>0.87</td>
</tr>
<tr>
<td>None</td>
<td>0.83</td>
<td>0.92</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* One-tenth-ml aliquots of antisera diluted to 1:16 in MEM were absorbed with an equal volume of brain homogenates.

Average values of duplicate samples from a representative experiment. Duplicate values agreed within 5%.
Table 3  
Cytotoxic activity of anti-DBLA-6 serum on rat leukemias and lymphomas

<table>
<thead>
<tr>
<th>Cell</th>
<th>Origin of tumor</th>
<th>Mode of induction</th>
<th>Morphological classification by May Giemsa staining</th>
<th>ALS CI</th>
<th>Cytotoxic titers</th>
<th>RF with GPRBC</th>
<th>Slg a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lymphoid cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anti-DBLA-6</td>
<td>Anti-RSTA</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>Thymus</td>
<td>G-MuLV</td>
<td>L</td>
<td>0.94</td>
<td>64</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>Lymph node</td>
<td>Thymus</td>
<td>G-MuLV</td>
<td>L</td>
<td>0.99</td>
<td>32</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>Neonatal spleen</td>
<td>Thymus</td>
<td>G-MuLV</td>
<td>L</td>
<td>0.95</td>
<td>32</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>AKR/J thymus</td>
<td>Thymus</td>
<td>LLVF</td>
<td>L</td>
<td>0.96</td>
<td>128</td>
<td>64</td>
<td>+</td>
</tr>
<tr>
<td>AKR/J thymus</td>
<td>Thymus</td>
<td>G-MuLV</td>
<td>L</td>
<td>0.99</td>
<td>128</td>
<td>64</td>
<td>+</td>
</tr>
</tbody>
</table>

Group 1
- WGT-13: Thymus, G-MuLV, L, 0.94, 64, 16, +, -
- WGT-15: Thymus, G-MuLV, L, 0.99, 32, 8, +, -
- WGT-20: Thymus, G-MuLV, L, 0.95, 32, 16, +, -
- WGT-25: Thymus, G-MuLV, L, 0.96, 128, 64, +, -
- WLT-6: Thymus, LLVF, L, 0.99, 128, 64, +, -

Group 2
- WFT-22: Spleen, F-MuLV, L, 0.98, 32, 8, -, -
- WFT-24: Spleen, F-MuLV, L, 0.97, 32, 8, -, -
- WFT-25: Spleen, F-MuLV, L, 0.80, 16, 8, -, -
- WRT-5: Lymph node, R-MuLV, L, 0.8, 4, 4, -, -
- WRT-6: Lymph node, R-MuLV, L, 0.90, 16, 8, -, -

Group 3
- DBLA-1: Bone marrow, BNU, U, 0.89, 64, 16, -, -
- DBLA-6: Bone marrow, BNU, U, 0.92, 32, 8, -, -
- DBLA-9: Bone marrow, BNU, U, 0.97, 64, 16, -, -

Group 4
- KNL-13: Bone marrow, BNU, U, 0.02, 16, 4, -, -

Group 5
- DBLA-10: Bone marrow, BNU, G, 0.10, -, -, -, -
- L1005: Bone marrow, BNU, G, 0.07, -, -, ND, ND

Group 6
- AL-804: Unknown, ENU, E, 0.10, -, -, ND, ND
- WFT-28E: Spleen, F-MuLV, E, 0.16, -, -, ND, ND
- WFT-29E: Spleen, F-MuLV, E, 0.04, -, -, ND, ND

Species-specific antigen (RSTA) was prepared by absorbing anti-DBLA-6 serum with AKR/J brain homogenate; the cytotoxicity of both anti-DBLA-6 and anti-RSTA sera were then examined in these 6 groups of rat leukemias. Both antisera were cytotoxic not only for DBLA-1, DBLA-6, and DBLA-9 (Group 3) but also for KNL-13 (Group 4) and all thymic (Group 1) and nonthymic (Group 2) lymphomas, while all myelogenous leukemias (Group 5) and erythroleukemias (Group 6) were not cytolyzed by these antisera. These results are summarized in Table 3.

DISCUSSION

DBLA leukemia lines were induced in female Donryu rats with BNU by Odashima et al. (14) in 1969. We previously reported the serological characterization of DBLA-1, DBLA-6, and DBLA-9 and that these leukemias lack both mature T- and B-cell antigens (10). In the present study, immunization of rabbits with "non-T, non-B" leukemia DBLA-6 cells elicited the production of antibodies which killed in the presence of guinea pig complement 70 to 90% of thymocytes and 40 to 50% of neonatal spleen cells. Normal lymphoid cells such as spleen, lymph node, bone marrow, and peritoneal cells were negative on the cytotoxicity test. However, a quantitative absorption analysis revealed that an antigen identified by anti-DBLA-6 serum is expressed on bone marrow and spleen cells in a very small amount. Such distribution of the antigen recognized by anti-DBLA-6 serum is very similar to that of Thy-1 antigen reported by several investigators in rats (1, 5). Furthermore, a cross-absorption test indicated that anti-DBLA-6 serum contains antibodies against at least 3 sets of antigenic determinants on rat thymocytes. One of these is a species-specific determinant. The second determinant is shared in rat and AKR/J mouse thymocytes or brain tissues (probably Thy-1.1). The third is a determinant cross-reacting among rat, mouse, and human brain tissues. Similar results have been reported in an analysis of rabbit anti-rat brain serum by Clagett et al. (2). Therefore, it is most probable that the antigen recognized by anti-DBLA-6 serum is identical to or closely related to rat Thy-1 antigen and that the RSTA recognized by AKR/J brain-absorbed anti-DBLA-6 serum is a species-specific xenogenic determinant. Recently, Goldschneider (4) isolated a "null" population of lymphocyte-like cells from the bone marrow of adult rats. Heteroantiserum to this "null" population (anti-BMLA) also reacted with bone marrow lymphocytes, cor-

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tical thymocytes, and neonatal spleen cells. Serological characterization of anti-BMLA serum revealed that this antiserum also recognized an antigen identical to or closely related to Thy-1 antigen (5). Taken together, it is highly probable that DBLA-6 is derived from such a “null” population of rat bone marrow cells.

The anti-DBLA-6 serum was next applied to the characterization of rat leukemias and lymphomas. We divided 19 rat leukemias and lymphomas into 6 groups by using 3 cell markers and morphological analysis. It is very interesting that anti-DBLA-6 serum was cytotoxic not only for DBLA-1, DBLA-6, and DBLA-9 (Group 3) but also for KNL-13 (Group 4) and all thymic (Group 1) and nonthymic (Group 2) lymphomas tested, while not cytotoxic for all myelogenous leukemias (Group 5) and erythroleukemias (Group 6) tested. Moreover, it was shown that thymic lymphomas induced by G-MuLV or LLVF have the same cell markers as do normal thymocytes and nonthymic lymphomas induced by F-MuLV or R-MuLV have same ones as do a subpopulation of neonatal spleen cells. This suggests the possibility that thymocytes and neonatal spleen cells are target cells for malignant transformation by respective MuLV. It has been reported that BNU has its main leukemogenic effect on bone marrow cells in rats (13). We have previously reported the serological characterization of BNU-induced leukemia DBLA-1, DBLA-6, and DBLA-9 (Group 3) and that these leukemias may be derived from lymphopoietic stem cells (10). In the present study, BNU-induced leukemia KNL-13 (Group 4) is of particular interest. KNL-13 is assumed not to be of lymphoid origin and to be more immature than were DBLA-1, DBLA-6, and DBLA-9 because KNL-13 reacted with anti-DBLA-6 serum but not with ALS. This observation may be interpreted as indicating a different stage of cell differentiation where leukemic transformation may have taken place. KNL-13 may be derived from a pluripotent stem cell and might be able to be designated as “stem cell” leukemia from antigenic and morphological characteristics. This proposal may be further supported by the recent report that rat pluripotent stem cells possess Thy-1 antigens or BMLA antigens (5). For the above-mentioned reasons, it is strongly suggested that the antigen identified by anti-DBLA-6 serum is expressed from “stem cell” leukemias to well-differentiated lymphomas along the lymphocyte differentiation pathway.

The preparation of anti-DBLA-6 serum is very easy and, as we reported, the antiserum is more specific for rat thymocytes than was rabbit anti-rat brain serum (11). Therefore, characterization of rat leukemias and lymphomas by anti-DBLA-6 serum in combination with other cell markers will contribute to the classification of rat leukemias and lymphomas. This work is now in progress in our laboratory.

REFERENCES

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