Establishment and Characterization of a Transplantable Rat Myelomonocytic Leukemia

Tetsuya Moriuchi, Tsuneyuki Oikawa, Takao Kodama, Hideo Yamaguchi, and Hiroshi Kobayashi

Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine, Kita-15-jo, Nishi-7-chome, Sapporo 060, Japan

ABSTRACT

A transplantable myelomonocytic leukemia was established from a leukemia of a WKA/Hok rat which had been inoculated with Rauscher virus at birth. The tumor grew in ascites form in normal syngeneic rats and, after the middle stage of i.p. transplantation, leukemia cells consisting of a mixed population of monocytic and granulocytic cells were observed in the peripheral blood. A complement-dependent cytotoxicity test failed to demonstrate Rauscher virus-related antigen on the tumor cell surface. Membrane marker analysis revealed that most of the tumor cells possessed receptors for both complement and neuraminidase-treated sheep RBC. More than 90% of ascitic tumor cells displayed phagocytic activity and a positive nonspecific esterase reaction. Serum from rats bearing this tumor contained high levels of muramidase. Ultrastructurally, the tumor cells resembled both immature and mature cells of the monocyte-macrophage series. On serial transplantation into the peritoneal cavity, the tumor displayed consistent differentiation from undifferentiated blast cells to monocytes and cells indistinguishable from granulocytes. The karyotype analysis revealed that the modal number of chromosomes of the tumor cells was 81, and no structural abnormalities of chromosomes were observed after quinacrine mustard staining. This transplantable leukemia will provide a useful experimental model for the study of granulocyte-monocyte differentiation and for human myelomonocytic leukemia.

INTRODUCTION

Acute myeloid leukemia is a neoplastic disease characterized by the proliferation of immature cells of myeloid origin. Because of the common origin of several hematopoietic cell lines, the leukemic process involves cells committed to granulocyte-monocyte differentiation, as well as those directed toward erythropoiesis and probably megakaryocytopeniosia (3, 21). There is a wide range of morphological appearances in human acute myeloid leukemia. Attempts to categorize the heterogeneity have been made on the basis of cytochemical staining, degree of differentiation, and immunological cell markers (2). These studies have been reported to provide prognostic guidance and to be correlated with response to therapy (1).

Although myelomonocytic leukemia is a well-recognized entity in human acute myeloid leukemias, no well-characterized model of the disease exists in inbred rats for basic analysis of the development of this leukemia and for its treatment. Furthermore, until now, no rat cell lines with macrophage properties have been available for biological or immunological experiments. In the present paper, we describe the occurrence of myelomonocytic leukemia in a WKA/Hok rat, which had been given an injection of Rauscher virus as a newborn, and the subsequent behavior of that tumor on serial transplantation. The WRT-7 leukemia cells differentiated in vivo into the monocyte-macrophage series and granulocytes. Cell surface marker analysis demonstrated that most of the leukemic cells possess receptors for nSRBC which are specific cell markers for rat macrophages (15).

MATERIALS AND METHODS

Animals and Tumors. WKA/Hok rats were obtained from the Experimental Animal Center, Faculty of Science, Hokkaido University, Sapporo, Japan. Tumors used in the present experiment were: (a) Gross murine leukemia virus-induced lymphoma, WGT-20; (b) Friend lymphatic leukemia virus-induced lymphoma, WLFT-6; (c) Friend murine leukemia virus-induced lymphomas, WFT-2N, -22, and -24; (d) Rauscher murine leukemia virus-induced lymphomas, WRT-5 and -6; (e) 1-buty1-1-nitrosourea-induced leukemia, DBLA-6, KNL-13, and L1005; and (f) 1-ethyl-1-nitrosourea-induced leukemia, KNL-11. Characterization of these tumors has been reported previously (13, 14).

Virus. Rauscher virus was originally provided by Dr. K. Yokoro, Hiroshima University, Hiroshima, Japan. The virus was recovered by a modification of the method of Chenaille et al. (4) from the enlarged spleen of dd/N mice with Rauscher leukemia. Undiluted virus (0.20 ml) in phosphate-buffered saline (containing, g/liter, KCl, 0.20; KH_2PO_4, 0.20; NaCl, 8.00; and Na_2HPO_4·7H_2O, 2.16) was injected s.c. and i.p. into newborn WKA/Hok rats.

antisera. The same batches of antisera that were used in previous studies (13) were used here. Briefly, rabbit antilymphocyte serum was produced by giving rabbits i.v. injections of WKA/Hok lymph node cells 3 times at 2-week intervals followed by bleeding 1 week after the last injection. The antisera were absorbed twice with an equally packed volume of syngeneic KMT-17 fibrosarcoma cells and once with an equally packed volume of WKA/Hok brain homogenate to absorb contaminating anti-Thy-1 antibodies. Rabbit anti-T lymphocyte serum was prepared by the method of Ishi et al. (11). Rabbit anti-B lymphocyte serum was produced by absorbing antilymphocyte serum with WKA/Hok thymocytes. Anti-DBLA-6 serum was obtained by immunization of rabbits with DBLA-6 cells 3 times i.v. at 2-week intervals and by bleeding them at 1 week after the last injection. The antiserum was absorbed 3 times with an equally packed volume of syngeneic AH-66 hematoma cells. This anti-DBLA-6 serum recognizes the antigen which is identical to or closely related to rat Thy-1 antigen (14). Antiserum against Rauscher virus-related antigens (anti-RV) was produced by 6 weekly s.c. inoculations of WKA/Hok rats with 5 x 10^7 WRT-5 cells.

Cytotoxicity Test. Equal volumes (0.05 ml) of antiserum at doubling dilutions, a cell suspension (5 x 10^6/ml) in Eagle’s minimum essential medium, and guinea pig complement were incubated at 37° for 45 min and washed in cold Eagle’s minimum essential medium. The proportion

1 Supported in part by a research grant for cancer research from the Ministry of Education in Japan.
2 Present address: Department of Microbiology and Public Health, Michigan State University, East Lansing, Mich. 48824.
3 To whom requests for reprints should be addressed.

Received September 27, 1982; accepted August 9, 1983.
of dead cells was determined by trypan blue staining. More than 200 cells were counted in each sample. The cytotoxicity index was calculated as:

\[
(c - f) \times 100/c
\]

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%.

Rosette Assay for the Binding of YC and nSRBC. The YC rosette method described by Rivero et al. (17), based on the use of baker's yeast particles previously treated with human serum, was used to detect complement receptors on cell membranes. Complement-coated yeasts were suspended in fetal calf serum and adjusted to \(3 \times 10^8\) cells/ml. The nSRBC rosette method described previously (15) was used to identify rat monocyte-macrophage series. Sheep erythrocytes were purchased commercially. They were washed 3 times in HBSS and adjusted to a 10% suspension in HBSS. The erythrocytes were incubated with Vibrio cholerae neuraminidase (5 units/ml of suspension; Behringwerke AG) for 30 min at 37°. After incubation, the cells were washed 3 times and resuspended in HBSS containing 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) at a final concentration of \(3 \times 10^8\) cells/ml.

Rosette formation was performed by the modified method of Tachibana and Ishikawa (19). One \(\mu\)l of cell suspension (\(3 \times 10^8\) cells/ml) was added to the poly-L-lysine (Type 1; Sigma Chemical Co., St. Louis, Mo.)-coated wells of a Falcon 3094 plastic microtest plate, and the plate was centrifuged at 150 \(\times g\) for 3 sec (Kubota KS-4000, Tokyo, Japan). Five \(\mu\)l of indicator cell suspension (\(3 \times 10^8\) cells/ml) were then added to the wells and again centrifuged at 150 \(\times g\) for 3 sec. The plate was then inverted and allowed to stand in this position for 10 min at room temperature. The rosette-forming cells and unreacted cells were counted in this position under a microscope. Usually, 4 to 5 wells were used for each sample tested, and 100 to 200 cells from each well were examined. Cells binding more than 3 indicator cells were considered positive.

Cytological Examination. Blood and ascitic tumor cells were smeared on glass slides and stained with May-Grünwald-Giemsa solution. Peroxidase, periodic acid-Schiff, and nonspecific esterase reactions were also tested with cell smears.

Phagocytic Activity. India ink was added to a cell suspension (5 \(\times 10^8\) cells/ml) at a final concentration of 0.5% for the phagocytosis test of cells. These cells were stained with May-Grünwald-Giemsa after 1 hr of incubation at 37°, and the number of cells with phagocytized carbon particles was counted under a microscope.

Assay of the Muramidase Activity. Muramidase activity was determined by a modification of the lysis plate method (16) in lysoplates containing 1% agar, 0.067 M sodium phosphate buffer (pH 6.6), 0.05 M NaCl, and spray-dried cell powder of Micrococcus luteus (Miles Laboratories, Inc., Elkhart, Ind.) as a substrate. After 24 hr at 25°, the diameter of the clear zone formed by the lysis of bacteria was measured. Hen egg white lysozyme (Seikagaku Kogyo, Japan) was used in each experiment as a standard, and 1 unit of lysozyme activity was defined as equivalent to that of 1 \(\mu\)g of hen egg white lysozyme.

Electron Microscopic Examination. Ascitic tumor cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, for 1 hr at 4°. The specimens were then postfixed in cold 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.5, for 1 hr. They were embedded in Epon 812. Thin sections prepared with an LKB Ultrotome were stained with lead and uranyl acetate and examined in a Hitachi H-12 electron microscope.

Karyotype Analysis. Chromosome specimens from WRT-7 tumor cells maintained in syngeneic rats in ascites form were directly prepared with the usual air drying method. Cells treated with a hypotonic solution (0.075 M KCl) containing colchicine (0.2 \(\mu\)g/ml), for 30 min at 37°, were fixed with several changes of acetic acid:methanol (3:1) and spread on clean glass slides. Giemsa staining was used to determine the modal chromosome number, and quinacrine mustard staining was used to analyze chromosome abnormalities. Karyotypes were arranged with the photographed metaphase plates according to the standard nomenclature for rat chromosomes (6).

RESULTS

Occurrence of Leukemias in Rauscher Virus-infected Rats. During a study of the characterization of rat leukemias and lymphomas, we examined blood samples of five 36-week-old rats which had been given injections of Rauscher virus within 48 hr after birth. Two rats were revealed to have lymphoblastic leukemia, and one female rat showed a marked monocytosis. A submaxillary lymph node was excised aseptically from the rat with monocytosis, and a cell suspension was prepared. Cytotoxicity tests revealed that approximately one-half of the cells were not of lymphoid origin. Surface marker analysis showed that 51% of the cells had receptors for complement, and 46% of the cells formed rosettes with nSRBC. Cytotoxicity examinations were performed on peripheral blood smears. They showed that 89 and 99% of the cells were negative in myeloperoxidase and periodic acid-Schiff reactions, respectively. These results are summarized in Table 1. This female rat was killed, and an autopsy was performed. The number of circulating leukocytes at autopsy was 107,500/cu mm, most of them being mononuclear cells (Fig. 1a). The spleen and the liver were not enlarged. The thymus was atrophied, but lymph nodes were markedly enlarged (total weight, 5 g).

Establishment of a Transplantable Myelomonocytic Leukemia. A cell suspension prepared from enlarged lymph nodes in the original rat was transferred i.p. to both syngeneic normal rats and Rauscher virus-tolerant rats (12). Unexpectedly, 4 weeks later, leukemia developed in all normal rats as well as in Rauscher virus-tolerant rats. The leukemia grown in normal rats was designated WRT-7. Ultrastructurally, the WRT-7 cells had an ovoid or irregularly shaped nucleus. Nucleoli were noted in about one-half of the cells. The cytoplasm contained numerous free ribosomes, varying numbers of small dense granules, and many vacuoles. The irregular ruffled cell margin was apparent with numerous microprojections (Fig. 2). Budding C-type virus

<table>
<thead>
<tr>
<th>% of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRT-7 (primary leukemia)</td>
</tr>
<tr>
<td>Lymph node cells</td>
</tr>
<tr>
<td>Anti-lymphocyte serum</td>
</tr>
<tr>
<td>Anti-T</td>
</tr>
<tr>
<td>Anti-B</td>
</tr>
<tr>
<td>Anti-DBLA-6</td>
</tr>
<tr>
<td>YC rosette</td>
</tr>
<tr>
<td>nSRBC rosette</td>
</tr>
<tr>
<td>Peripheral blood cells</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>Peroxidase</td>
</tr>
<tr>
<td>WRT-7-1*</td>
</tr>
<tr>
<td>Asciate cells</td>
</tr>
<tr>
<td>Anti-lymphocyte serum</td>
</tr>
<tr>
<td>Anti-T</td>
</tr>
<tr>
<td>Anti-B</td>
</tr>
<tr>
<td>Anti-DBLA-6</td>
</tr>
<tr>
<td>Anti-RV</td>
</tr>
<tr>
<td>YC rosette</td>
</tr>
<tr>
<td>nSRBC rosette</td>
</tr>
<tr>
<td>Phagocytic activity</td>
</tr>
<tr>
<td>Nonspecific esterase</td>
</tr>
</tbody>
</table>

* Passage number.

NOVEMBER 1983

5479
particles were often seen. Cytological examination of the ascites smear showed a heterogeneous collection of cells including basophilic blast cells, immature and mature monocytes, myelocytes, and metamyelocytes. Cytochemical and biological examinations of ascites tumor cells revealed positive nonspecific esterase reactions and phagocytic activity for carbon particles in most of the ascites tumor cells. All cytotoxicity tests using antilymphocyte, anti-T, anti-B, and anti-RV sera were negative in ascites tumor cells. However, surface marker analysis revealed that more than 90% of the cells possessed receptors for both complement and nSRBC. These results are summarized in Table 1. Serum levels of muramidase activity in WRT-7-bearing rats were 4 to 64 times higher than those in normal rats.

**Differentiation of the WRT-7 Leukemia Cells in Vivo.** Ascites tumor cells were smeared for staining with May-Grünwald-Giemsa at 3-day intervals for 4 passage generations. Seven days after i.p. transplantation of $1 \times 10^8$ tumor cells, an increase of blast cells with multiple nucleoli was evident and, on Day 9, approximately 80% of ascites cells consisted of blast cells and immature cells with large basophilic cytoplasmic vacuoles. By Day 12, the percentage of blast cells had decreased, and the differentiation of immature cells to the monocytic series was observed. On Day 21, more than 90% of ascites cells were mature cells of the monocyte-macrophage series with abundant gray-blue cytoplasm, many cytoplasmic vacuoles, and indented or folded nuclei (Fig. 1b). The number of mature monocyte-macrophage-type cells increased up to the time of death. The interesting feature of the WRT-7 tumor, however, was that its cells appear to differentiate to eosinophilic granulocytes. Coincident with the increase of blast cells on Day 9, eosinophilic myelocytes and metamyelocytes appeared. By Day 12, immature and mature eosinophilic granulocytes composed about 8% of the ascitic cells. Abnormally vesiculated eosinophils and atypical small eosinophils with pyknotic nuclei were often seen in this period. By Day 15, immature eosinophilic granulocytes were rarely observable, and concomitantly mature eosinophils had decreased in number. By Day 18, eosinophilic granulocytes were undetectable. Neutrophilic myelocytes and metamyelocytes were rarely seen (Fig. 3). The process of differentiation of monocytes and granulocytic cells was reproducible in different passage generations.

**Rosette-forming Capacity of Various Rat Leukemias and Lymphomas with nSRBC.** We have previously reported that nSRBC form rosettes specifically with rat macrophages (15). Therefore, we investigated whether nSRBC rosette formation is also useful for the characterization of rat monocytic leukemia. YC and nSRBC rosette formation was performed in various rat ascitic leukemias and lymphomas. Because normal peritoneal cells contaminate tumor cells, less than 15% of YC and nSRBC rosette formation is considered to be negative in this experiment. Both YC and nSRBC rosette formation was negative in thymic lymphoma, lymphatic leukemia, stem cell leukemia, and erythroleukemia. YC rosette formation was positive in nonthymic lymphoma and granulocytic leukemia, but nSRBC rosette formation was negative in them. Only myelomonocytic leukemia, WRT-7, showed positive reaction with both YC and nSRBC (Table 2). A small population of the WRT-7 cells with a morphology of immature eosinophilic granulocyte did not form rosettes with nSRBC.

**Karyotypic Analysis of WRT-7 Leukemia Cells.** The number of chromosomes within cells was counted using 50 well-spread Giemsa-stained metaphase plates. The modal chromosome number of the WRT-7 leukemia was 81 with some variability. No structural rearrangements of chromosomes were observed as far as the quinacrine mustard-banding pattern of 10 metaphase plates was analyzed (Fig. 4).

**DISCUSSION**

Although myelomonocytic leukemia is a well-known entity in humans, induction of this leukemia has been very difficult in experimental animals, especially in rats. In mice, a transplantable myelomonocytic leukemia and a few cell lines with macrophage properties have been reported (7, 20). The classification of myelomonocytic leukemia in animals has thus far been based on morphological and cytochemical criteria. Membrane markers are not yet routinely used for its characterization. In the present study, several antigenic and surface markers were used for the characterization of the WRT-7 leukemia in combination with morphological and cytochemical analyses. Myelomonocytic leukemia was first noticed by a marked monocytosis consisting of a mixture of monocytic and myelocytic cells. This diagnosis was strongly supported by the high percentage of nonspecific esterase-positive cells and the high level of serum muramidase activity. We have previously characterized rat leukemias and lymphomas by using a panel of membrane markers and classified them into 6 groups (13). The membrane phenotype of the WRT-7 resembles granulocytic leukemia, i.e., it possesses complement receptor but lacks all other antigenic markers used. The final conclusion was made from nSRBC rosette formation. As we have reported previously, rat macrophages form rosettes specifically with nSRBC (15). The WRT-7 leukemia cells demonstrated receptors for both nSRBC and complement, while 2 granulocytic leukemias, DBLA-10 and L1005, had only complement receptors. Thus, nSRBC rosette formation provides a useful tool for distinguishing granulocytic and monocytic cells.
which can sometimes be difficult, especially in myelomonocytic leukemia.

In rats, Rauscher virus induces extrathymic lymphomas exclusively, and most of these are rejected by the host immune response against Rauscher virus-associated cell surface antigens (12) when transplanted into normal syngeneic rats. In the present work, we failed to demonstrate the presence of Rauscher virus-associated cell surface antigen on WRT-7 leukemia cells by a complement-dependent cytotoxicity test. This result is in accord with the fact that WRT-7 can grow in normal syngeneic rats. These findings lead to 3 possible explanations as to the induction of this leukemia, i.e., this leukemia was induced: (a) spontaneously; (b) by Rauscher virus; or (c) by another virus contaminating the Rauscher virus. The first explanation is most unlikely, because no spontaneous leukemia has been observed in WKA/Hok rats, and the WRT-7 leukemia developed at the same time as lymphomas did in other rats which had been given injections of the same batch of Rauscher virus simultaneously. The third possibility cannot be ruled out, because the virus was recovered from the enlarged spleen of dd/N mice with Rauscher disease. More detailed analysis of the cell surface antigens will be required to determine the causative agent of this leukemia.

The WRT-7 may be a good model for the study of cell differentiation of the monocyte-granulocyte series, because the WRT-7 leukemia repeatedly differentiated from undifferentiated blast cells to cells of monocytic-granulocytic series in the peritoneal cavity after serial transplantation. Several myeloid leukemia cell lines have been induced to differentiate to macrophage and/or granulocytes in vivo and in vitro under the influence of various inducers (5, 9). However, the differentiation of myeloid leukemia cells has always been into 2 types of cells, i.e., macrophage and neutrophilic granulocyte, although cultures of rat chimeras with cells containing granules with a structure similar to eosinophil granules have been reported (10). Therefore, one of the questions raised by the WRT-7 leukemia concerns the origin of the eosinophilic granulocytes. The possibility that only the monocytes are truly malignant cells and that the granulocytes are normal host cells participating in a leukemoid response to the tumor is very unlikely based on the following facts. (a) Normal eosinophilic myelocytes and metamyelocytes never appear in ascites. (b) Eosinophilic myelocytes and metamyelocytes are observed in ascites only at the end of early stages and disappear completely in the late stage of transplantation. (c) When immature eosinophils are disappearing, abnormally matured eosinophils appear. Another possibility that immature eosinophilic cells might be monoblasts or degenerating monocytes is also unlikely; because they did not form rosettes with nSRBC. Although this circumstantial evidence favors tumor derivation of the eosinophilic cells, further analysis will be necessary to determine the origin of the eosinophilic cells.

It is an open question as to whether the fundamental lesion in this myelomonocytic leukemia occurred in a pluripotent hematopoietic stem cell or in a committed stem cell that has more restricted developmental pathways. The ability of the WRT-7 cells to undergo maturation without exogenous inducers indicates that the WRT-7 cells have receptors for host differentiation-inducing factors that normally trigger committed stem cells to differentiate. Therefore, abnormal accumulation of the leukemic cells in vivo cannot be explained by a blockade or alteration of the receptors for differentiation-inducing factors on these leukemic cells as has been proposed by others (18). One possible explanation is that committed stem cells may be overproduced owing to a loss of a negative feedback inhibition mechanism which regulates normal hematopoiesis of cells of the granulocyte-monocyte series, leading to an abnormal accumulation of leukemic cells. We have recently succeeded in establishing an in vitro cell line of the WRT-7 leukemia which can be induced to differentiate to macrophages and granulocytes by chemical inducers (8). Both in vivo and in vitro cell lines of the WRT-7 leukemia provide useful models for the study of differentiation of normal and neoplastic hematopoietic cells. Furthermore, they will provide a useful experimental model for human myelomonocytic leukemia.

REFERENCES
Fig. 1. May-Grünewald-Giemsa-stained smear. a, ×400; b, peripheral blood picture of primary leukemia. b, ×8000.

Fig. 2. Electron microscopy of WRT7 leukemia cells in ascites. ×8000.
Fig. 3. Analysis of W17 leukemic-bearing rat a, undifferentiated blast cells (6 days); b, promyelocytes (6 days); c, eosinophilic myelocytes (8 days); d, myeloblasts (8 days); e, neutrophilic myelocytes (12 days); f, monocytes (12 days); g, megakaryocytes (12 days); h, erythroblasts (12 days).

Fig. 4. Quinacrine mustard-stained chromosomes from W17 leukemic cells.

NOVEMBER 1983
Establishment and Characterization of a Transplantable Rat Myelomonocytic Leukemia

Tetsuya Moriuchi, Tsuneyuki Oikawa, Takao Kodama, et al.


**Updated version**

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/11/5478