Isolation and Characterization of a Spontaneous Lymphocytic Leukemia (L-76) in the Strain 2 Guinea Pig

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ABSTRACT

A partial characterization of a transplantable guinea pig leukemia (L-76) that arose spontaneously in a young female strain 2 animal is described. The leukemia appears to be histologically, pathologically, and karyologically similar to the LjC lymphoblastic leukemia, which also arose spontaneously 24 years earlier. The presence of C3 receptor sites on the surface of the leukemia indicates it to be of B-cell origin. Although the L-76 leukemia originated in a strain 2 animal, it also produces a rapidly progressing, lymphoblastic leukemia in strain 13, Hartley, and strain 2 × strain 13 F, hybrid adult guinea pigs. An inoculation of 2 × 10⁶ blast cells obtained from peripheral blood induced a stem cell leukemia in the strain 2 host within 12 days, with white blood cell counts of 2 to 3 × 10⁶ cells/cu mm at expiration. Titration data indicated that as few as 10 cells injected s.c. were capable of transmitting a systemic disease, which reached a terminal phase by 40 days. Pathological examination indicated involvement of the entire hematopoietic system, with almost all organs infiltrated by massive concentrations of neoplastic lymphoblasts. Treatment of the leukemia with Cytoxan (cyclophosphamide), 100 mg/kg, provided a remission period of 3 to 4 weeks later.

INTRODUCTION

The LjC leukemia was isolated in the 1950's by Congdon and Lorenz (2) as 1 of a series of spontaneous and radiation-induced leukemias observed in the NIH guinea pig colony over a period of years. This leukemia, which by this time has evolved into a group of sublines based on subtle genetic and immunological differences, is the only transplantable guinea pig leukemia currently available (8). Although the LjC leukemia has been of great value in a variety of immunological studies, the lack of additional recently isolated leukemias has hampered efforts in areas such as the tumor virology of the guinea pig and has limited generalizations on the etiology of leukemia in the guinea pig.

In this report we describe a spontaneous lymphoblastic leukemia recently isolated in a strain 2 guinea pig (designated L-76 leukemia). Results on its transplantability, histology, pathology, karyology, and other biological characteristics will be presented in addition to comparing these properties to those of the LjC-EN leukemia.

MATERIALS AND METHODS

Animals. Inbred strain 2-N guinea pigs were obtained from the Frederick Cancer Research Center Animal Farm, Frederick, Md., while strain 13, random-bred Hartley, and strain 2 × strain 13 F, hybrid guinea pigs were obtained from herds maintained at the NIH Animal Farm, Bethesda, Md. Animals were housed in stainless-steel cages and fed Wayne guinea pig chow and water ad libitum and kale twice weekly. All animals were age matched and weighed 300 to 400 g when experiments were initiated.

Leukemias. The history and biological properties of the LjC (EN subline) leukemia have been previously described (4, 6, 7, 8, 10, 13, 18). The LjC-EN subtype was kindly supplied by Dr. I. Green (NIH, Bethesda, Md.). The L-76 leukemia arose spontaneously in 1976 in a 5.5-month-old female strain 2 guinea pig. At necropsy the disease involved the entire hematopoietic system with generalized enlargement of the spleen and lymph nodes, typical of an acute leukemia. A peripheral WBC was greater than 200,000/cu mm. Blood and tissue specimens were collected and shipped to us on ice by Dr. R. Hong from the Frederick Cancer Research Center Animal Farm. Upon arrival the blood and a spleen homogenate were immediately injected into normal strain 2 guinea pigs. These recipient guinea pigs eventually died of leukemia 3 to 4 weeks later.

The L-76 leukemia is maintained as a transplantable cell line in strain 2 guinea pigs and is passaged serially every other week as a s.c. injection of 2 × 10⁶ viable leukemic blast cells obtained from peripheral blood. Material for in vivo transplantation was obtained from animals displaying WBC greater than 100,000/cu mm. Peripheral blood was collected via a cardiac puncture into heparinized syringes (5 units/ml) and diluted 1:3 with 0.01 M PBS, pH 7.2. Separation of blast cells was performed by centrifugation on a Ficoll-Hypaque gradient (1). After removal from the gradient, the cells were washed 3 times in cold PBS and either utilized the same day or preserved by storage in liquid nitrogen. Viability was generally greater than 95% for fresh cells and 85% for thawed cells. The material used for all in vivo and in vitro studies was obtained from leukemic...
strain 2 animals between the first and tenth serial transplant generations. Survival times were recorded following either a s.c. or an i.d. injection of different concentrations of L-76 or L2C-EN blast cells into strain 2, 13, Hartley, and F1 hybrid adult guinea pigs.

Immunization Procedures. Strain 2 guinea pigs were initially given injections in all 4 footpads of 4 x 10^7 L2C-EN or L-76 irradiated blast cells (6000 R generated by a Westinghouse Quadroconex X-ray machine; 200 kV constant potential; 15 ma; half-value layer, 0.9 mm copper) emulsified in CFA. Animals were subsequently boosted 2 weeks later with an i.d. injection of 2 x 10^6 viable leukemic cells of syngeneic origin.

Karyotype Analysis. Karyotypes were obtained from fresh peripheral blood samples drawn from leukemic guinea pigs. The technique used for chromosome examination was basically the same as that used by Whang-Peng et al. (19). Chromosome preparations were made by the air-dry method and were identified by banding patterns established by the Paris Conference (12). Analyses of chromosome numbers and karyotypes for the L-76 leukemia cells were made from photomicrographs of G-banded metaphase chromosomes and were compared to chromosomes obtained from normal guinea pigs.

Drugs. Cytoxan (CY) and MECCNU were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. Cytoxan was dissolved in 0.9% NaCl solution (pH 7.0), while MECCNU was solubilized in an ethanol-Emulphor vehicle and then further diluted in a 0.9% NaCl solution as previously described (13, 14). Both agents were administered i.p. in a constant volume of 0.001 ml/g of body weight.

Pathology. Infected guinea pigs bearing the transplantable tumor were sacrificed at a time near death, and samples from various tissues and organs were excised and fixed in neutral formalin. Sections were routinely stained with hematoxylin and eosin. Peripheral blood smears were stained with Wright’s solution for histological observation and periodic acid-Schiff and Sudan black for examination of peroxidase activity.

Assay for E-Rosette- and EAC Rosette-forming Cells. The rosette assay was used to detect the presence of specific antigen receptors on the surface of the L2C-EN and L-76 leukemias. Formation of E-rosettes was accomplished with rabbit RBC (17). The detection of lymphocyte receptors on both leukemias that are active for and bind to comple

Assay of RDDP. RDDP activity was measured in the presence of the template: primer poly(rC)oligo(dG)12-18. The complete reaction mixture in 100 µl consisted of 40 mM Tris-HCl (pH 7.8), 10 mM MgCl2 or 0.4 mM MnCl2, 10 mM KCl, 0.03% dithiothreitol, 0.1% Triton X-100, 5 µg poly(rC)oligo(dG)12-18, and 12.5 µCi [3H]GTP (20 Ci/mmol). Samples to be tested (plasmas from a variety of L2C sublines and L-76 plasmas) were clarified by centrifugation at 10,000 x g for 10 min at 4°C. These fluids were then layered over a 2.5-m1 cushion of 35% glycerol and centrifuged in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 40,000 rpm for 90 min. Pellets were resuspended in 100 µl of 0.01 M Tris (pH 7.8) and 50 µl were used in each assay.

Electron Microscopy. Leukemic guinea pigs were sacrificed, and the tissues were immediately removed, minced into approximately 1-cu mm pieces, and placed in 2.5% glutaraldehyde in PBS for 1 hr. WBC were isolated from peripheral blood as described above, pelleted, and resuspended in the glutaraldehyde fixative. After 2 to 5 min, the cells were replicated in Microfuge (Beckman Instruments, Inc.) tubes and then removed to vials, where they were suspended in fresh glutaraldehyde for 1 hr. After all samples were washed for 1 hr in PBS, they were postfixed in 1% osmium tetroxide in phosphate buffer, washed 1 hr in distilled water, stained overnight in 2% uranyl acetate in 50% ethanol, and dehydrated through a graded series of ethanol and propylene oxide. Blocks were embedded in Epon-Araldite, and ultrathin sections were stained with lead citrate and uranyl acetate and examined in a Siemens 101 electron microscope fitted with a 50-µm objective aperture.

RESULTS

Dose Response in Strain 2 Guinea Pigs. Strain 2 guinea pigs were given injections of various doses of either L2C-EN or L-76 leukemia s.c. to determine the interval between the time of leukemia injection and the death of the animal. Table 1 indicates that the duration of survival is a function of the number of cells initially injected for both leukemias. Both the L2C-EN and the L-76 leukemias disseminate widely, with blast cells appearing in the peripheral blood very early. There was no spontaneous recovery in any of the animals once the diseases became systemic following a s.c. injection.

<table>
<thead>
<tr>
<th>No. of leukemia cells injected</th>
<th>L2C-EN s.c.</th>
<th>L2C-EN i.d.</th>
<th>L-76 s.c.</th>
<th>L-76 i.d.</th>
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<tr>
<td>MST (days)</td>
<td>S/T</td>
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<td>MST (days)</td>
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<tr>
<td>10^6</td>
<td>&gt;60</td>
<td>5/5</td>
<td>ND</td>
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<td>&gt;60</td>
<td>2/5</td>
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<td>10^3</td>
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<td>10^1</td>
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S/T, number of survivors at 60 days per total number given injections; ND, not determined.

Immunofluorescent Assay for the Detection of Membrane Immunoglobulin. An assay initially described by Forni et al. (4) was used in this study. Lymphocytes were washed and treated directly with fluorescein-conjugated rabbit anti-guinea pig immunoglobulin at 4°C for 30 min. The cells were washed 3 times in PBS, resuspended in 0.1 ml of 50% glycerol-PBS, mounted on siliconized slides, and examined under a UV microscope (American Optical Co., Buffalo, N. Y.).
A minimum of $1 \times 10^4$ L\textsubscript{C}-EN blast cells injected s.c. was required to produce 100% mortality in the strain 2 guinea pigs, while as few as 10 L-76 cells were completely lethal under similar conditions, reaching a terminal phase by 40 days.

A s.c. injection of $1 \times 10^6$ cells for either leukemia led to the death of the animals by 15 to 16 days, but guinea pigs given s.c. injections of $1 \times 10^4$ L\textsubscript{C}-EN cells survived an average of 33 days, with 100% tumor take, as compared to a MST of 23 days and 100% tumor take for animals given s.c. transplants of $1 \times 10^4$ L-76 cells (Table 1). This difference indicates a higher malignant potential associated with the L-76 leukemia. Additional evidence suggesting an increased lethality for the L-76 leukemia was derived from experiments in which animals were given i.d. injections of either of the 2 leukemias. Table 1 further indicates that, of 5 animals given transplants of $1 \times 10^3$ L\textsubscript{C}-EN cells i.d., 4 survived. These animals were subsequently refractory to a further challenge with $1 \times 10^6$ viable L\textsubscript{C}-EN leukemia cells. However, when 5 guinea pigs were inoculated i.d. with a similar dose of $1 \times 10^3$ L-76 cells, all animals developed a fatal systemic disease within 30 days.

The growth of the L\textsubscript{C}-EN and L-76 leukemias in different guinea pig strains. Strain 2, strain 13, Hartley, and F\textsubscript{1} hybrid guinea pigs were given s.c. injections of L\textsubscript{C}-EN or L-76 leukemia blast cells. Strain 2 and F\textsubscript{1} hybrid animals inoculated with $2 \times 10^6$ L\textsubscript{C}-EN leukemia cells all died, with a MST of 12 to 14 days (Table 2). Although not indicated in Table 2, up to $2 \times 10^7$ L\textsubscript{C}-EN leukemia cells were rejected by all strain 13 and Hartley guinea pigs. Transplantation of $2 \times 10^6$ L-76 leukemia cells into strain 2, strain 13, and F\textsubscript{1} hybrids induced a generalized leukemia within 12 to 14 days with WBC of 2 to 3 x 10\textsuperscript{9} cells/cu mm at expiration. Growth of the L-76 leukemia in Hartley guinea pigs, however, was observed only in animals with alloantigens of B1 specificity. In all instances successful transplantation occurred when spleens, lymph nodes, peripheral blood, or localized tumor tissue derived from a leukemic strain 2 host were used. The MST in permissive Hartley guinea pigs given injections of $2 \times 10^6$ L-76 blast cells was slightly prolonged (18 days) when compared to survival times in strain 2, strain 13, and F\textsubscript{1} hybrid animals given injections of a similar concentration (Table 2).

Histopathology. Peripheral blood cells from L-76 leukemic animals near death were characteristic of lymphatic leukemia (Figs. 1 and 2). The neoplastic cells (diameter, 14 to 20 \textmu m) exhibited a smooth surface. Cytoplasm was scant and basophilic. The round to oval nuclei (diameter, 12 to 16 \textmu m) had prominent pleomorphic nucleoli similar to those of normal basophilic stem cells. The leukemic cells contained numerous small cytoplasmic vacuoles that were stained by Sudan black (indicating the presence of lipid) and osmium. Cytoplasmic peroxidase and esterase reactions were negative in all blast cells tested.

Virtually all of the organs that were removed from leukemic animals exhibited neoplastic involvement, but the thymus and marrow cells showed little involvement. The Peyer's patches were totally replaced by tumor cells and greatly enlarged. Metastases were composed of densely packed and fairly uniform lymphoblasts. However, in the areas of tumor growth benign-appearing histiocytes were interspersed between the neoplastic cells, and on microscopic observation they gave the typical "starry-sky cells" appearance, as seen in Burkitt's lymphoblastoma (Fig. 3). As observed in Fig. 4, mitotic figures were numerous, and tumor lesions were extensive in the liver (Fig. 5), lymph nodes, spleen, pancreas, and kidney. Relatively few neoplastic lesions were found in the lungs.

Karyotype Analysis of the L-76 Leukemia. Karyotype analysis of the L-76 leukemia revealed a modal chromosome number of 64 with 2 X chromosomes. All metaphases examined displayed an additional chromosome referred to as M1 since it was nearly metacentric. Other markers were also observed on the second chromosome pair, which consisted of 1 normal member and 1 with a terminal deficiency of the long arm (2q-). Another marker was identified in the third chromosome pair which contained a normal member and a deletion in the short arm (3p-). These 3 marker chromosomes (M1, 2q-, and 3p-) were found in 100% of the leukemic cells examined.

Immunogenicity Studies of Tumor-associated Transplantation Antigens. Animals were immunized with $4 \times 10^7$ irradiated leukemic blast cells emulsified with CFA by inoculation into all 4 footpads to determine the immunogenicity of the L\textsubscript{C} and L-76 leukemias in strain 2 guinea pigs. All animals were challenged i.d. with $2 \times 10^6$ viable cells 2 weeks later. Total inhibition of leukemic growth was observed only in those animals that were immunized with L\textsubscript{C}-EN leukemia and challenged with the same immunogen.
Expression of E-Rosette and EAC Rosette Receptors. Membrane markers were characterized on the L2C-EN and L-76 leukemias for the presence of lymphocyte receptors to rabbit erythrocyte and the third component of complement. Control assays indicated a large percentage of normal guinea pig lymph node and thymus cells exhibiting E-rosettes. Among normal cells tested only lymph nodes demonstrated complement receptors as indicated by EAC rosette formation (Table 4). A minimal number of E-rosettes were observed on the L2C-EN and L-76 leukemias, while EAC rosette receptors were present at high levels on both tumor cells (89% and 83%, respectively) (Table 4).

Immunofluorescent Staining for the Presence of Membrane Immunoglobulins. Using a direct immunofluorescent assay, surface immunoglobulins were detected on the membranes of both the L2C-EN and the L-76 leukemias. Approximately 90% of both leukemia cells showed positive fluorescence, while only 38% of normal lymph node cells revealed a positive stain.

Drug Therapy against the L-76 Leukemia. It was of particular interest to extend earlier studies on the effect of 2 chemotherapeutic agents on L2C-EN leukemia (13, 14) to include the L-76 leukemia. On Day 0 adult guinea pigs were administered 5 × 10⁶ L-76 blast cells in the inguinal area. Eleven days later, when the tumor size was between 8 and 12 mm and the average WBC was approximately 60,000/cu mm (about 85% blast cells), individual groups of animals were treated with either 1 or 2 courses of CY alone or in combination with various doses of MECCNU. As shown in Table 5, all control animals (Group 1) succumbed to the leukemia within 13 to 15 days. Treatment of leukemic guinea pigs on Day 11 with 1 or 2 courses of CY resulted in complete regression of the tumor as well as a complete hematological "remission" (5000 to 8000 WBC/cu mm) within 6 days. The effectiveness of the CY therapy, regardless of dose or regimen, was further verified by the prolongation of survival time as evidenced by an increase in the MST when compared to the untreated group. However, approximately 4 to 5 weeks following a drug-induced remission period, all CY-treated animals (Groups 2 and 3) began to exhibit elevated WBC’s (60,000 to 150,000/cu mm) and blast cells in the peripheral blood. Approximately 25% of these animals exhibited central nervous system involvement near death, but no distinct paralysis was apparent. This is in contrast to the marked hind leg paralysis observed in L2C-EN leukemic animals during relapse.

Treatment of leukemic animals (Day 11) with CY, 50 mg/kg, followed 1 week later with either 1 or 2 mg of MECCNU per kg (Table 5; Group 6 and Group 5, respectively) led to results similar to those for CY alone. Administration of MECCNU, 3 mg/kg, 1 week after CY (Group 4), however, resulted in 5 of 6 animals surviving beyond 90 days. No overt clinical or hematological signs of the leukemia were apparent during this period. To test the effect of MECCNU alone, we inoculated guinea pigs s.c. in the inguinal area with 5 × 10⁶ blast cells, and groups of leukemic animals were administered varying doses of MECCNU 11 to 12 days later. Under these conditions both 5 and 10 mg of MECCNU per kg elicited a complete clinical and hematological remission for a period of greater than 90 days (Table 5; Groups 8 and 7). Animals that received 2.5 mg of MECCNU per kg (Group 9) eventually succumbed to the leukemia within 34 to 40 days. Nevertheless, even at this low dose of drug the
effectiveness of MECCNU is reflected by a 153% increase in MST (38 days) as compared to the untreated control group (15 days). The response of L-76 leukemia to both CY and low-dose MECCNU chemotherapy is thus strikingly similar to the results obtained with the L,C-EN leukemia (13, 14).

**Reverse Transcription Assay of L-76 Plasma Samples.** Previous studies with different sublines of the L,C leukemia indicated that the "LC" subline but not others tested contained high levels of a Mg^{2+}-dependent reverse transcriptase associated with a particulate plasma fraction with a density in sucrose of 1.16 g/ml and containing RNA homologous to the RNA of the endogenous B-type RNA tumor virus of the guinea pig (3). Plasma samples from the L-76 leukemia grown in a variety of guinea pig strains were tested for RDDP activity. Repetitive tests indicated that L-76 leukemia does not contain detectable levels of either a Mg^{2+} or a Mn^{2+}-dependent reverse transcriptase. It appears that neither C-type nor B-type oncenvirus particles are present at measurable levels in L-76 plasma, and the activation of the B-type RNA tumor virus in guinea pigs with advanced LC leukemia remains a unique finding.

Examination of peripheral WBC, tumor, and tissues obtained from guinea pigs with L-76 leukemia by electron microscopy was carried out at both the first and second in vivo passages. Perhaps the most intriguing ultrastructural feature was the presence of relatively high levels of intracisternal A particles in both lymphoid tissues and the tumor cells. These virus-like particles have previously been detected in all sublines of L,C leukemia and were first observed in 1967 (11). They have also been observed at low levels in normal tissues of the guinea pig and in several established guinea pig cell lines.

Fig. 6 illustrates typical tumor cells as seen in the spleen of leukemia animals. The nuclei are highly pleomorphic and indented. This degree of indentation and segmentation is unusual and is not observed in any of the lines of the L,C leukemia. Furthermore, greater numbers of lipid vacuoles were observed with the L-76 leukemia than with the L,C sublines. Examination of different tissues of leukemic animals. Particles were never observed budding from external cell membranes, and it does not seem probable that the intracisternal A particles represent a virus with an oncogenic potential (3). Negative reverse transcriptase data suggested that neither type B nor type C retraviruses were present at detectable levels in the plasma of leukemic animals.

Earlier cytogenetic studies by Whang-Peng et al. (19) with various L,C sublines revealed chromosome anomalies on the L,C-BZ line (M1, 2q , 3p ) that were strikingly similar to the chromosome markers found on the L-76 leukemia.

Because of the striking pathological, histological, karyological, and electron microscopic similarities between the L-76 and L,C leukemias, additional studies are in progress to compare and analyze further the L-76 leukemia with selected sublines of the L,C leukemia. Various membrane markers and immunological parameters will be examined to determine whether any substantial differences exist between the 2 leukemias. In the absence of any known viral etiology for guinea pig leukemia, the similarities between these 2 leukemias, separated by 24 years and occurring in the same inbred strain of guinea pig, are particularly noteworthy.

**ACKNOWLEDGMENTS**

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**REFERENCES**

Characterization of a Guinea Pig Leukemia (L-76)


Fig. 1. Photomicrograph of L-76 lymphoblastic guinea pig leukemia obtained from peripheral blood. Giemsa, x 260.
Fig. 2. Photomicrograph of L-76 lymphoblastic guinea pig leukemia obtained from peripheral blood. Giemsa, x 1000.
Fig. 3. Metastatic lymphoblasts obtained from the Peyer's patches providing a starry-sky appearance. H & E, x 190.
Fig. 4. Mitotic figures in lymphoblasts obtained from Peyer's patches. Giemsa, x 400.
Fig. 5. Neoplastic lesions observed in the liver. H & E, x 260.
Fig. 6. Electron micrograph showing typical tumor cells as seen in the spleen of a leukemic guinea pig. × 8000.
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