Prolonged Survival in Long-Passage AKR Leukemia Using Chemotherapy, Radiotherapy, and Adoptive Immunotherapy

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SUMMARY

A chemoradioimmunotherapeutic model, with potential for clinical applicability, was tested in AKR mice bearing a long-passage lymphocytic leukemia. A temporary graft-versus-leukemia reaction was the key feature of the model. In the most successful experimental groups, graft-versus-host disease was circumvented and "cell cure" of a transplanted acute lymphocytic leukemia was achieved in at least 88% of the mice. Late mortality occurred in 58% of these mice and was due mainly to bone marrow and lymphoid aplasia of unknown cause. Appearance of spontaneous leukemia-lymphoma was significantly delayed or prevented in those mice that survived the first 100 days, and 20% lived beyond 21 months of age.

INTRODUCTION

Despite recent advances in the treatment of acute leukemia in man (13, 14, 16, 19), most patients die of recurrent leukemia (10). Chemotherapy usually is much less effective when used following relapse of leukemia after an initial remission and, with only occasional exceptions, sustained subsequent remissions do not occur (1). The experiments described below were performed in order to test a strategy that might have therapeutic applicability for the leukemic patient in whom conventional therapy has failed.

In brief, AKR mice bearing advanced acute lymphocytic leukemia were treated with a combination of chemotherapy, irradiation therapy, and adoptive immunotherapy using a transplant of bone marrow and lymph node cells from H-2-incompatible DBA/2 donors. After 6 days, the AKR mice were "rescued" from potentially lethal GVH disease by killing the DBA/2 cells using DBA/2-specific antiserum plus chemotherapy. Subsequently, bone marrow transplants from H-2-compatible CBA donors were administered for final hematopoietic reconstitution of the AKR mice. A significant proportion of the treated mice were "cured" of the transplanted leukemia and were long-term survivors.

The unique feature of the treatment plan consists of a temporary transplant of immunocompetent cells from unrelated donors for their adoptive immunotherapeutic effect. This approach is conceptually similar to that originally described by Boranic (4). Several modifications of Boranic's treatment plan have been introduced in order to simulate more closely problems encountered in clinical bone marrow transplantation for leukemia. The most important modification is the use of cells from allogeneic histocompatible donors for final hematopoietic reconstitution, rather than transplants of cells from syngeneic donors.

MATERIALS AND METHODS

Experimental Design. The model used is diagramed in Chart 1. Normal young AKR mice were given i.v. injections of 10^6 leukemic "blast" (large) cells obtained from spleens of AKR mice that had received similar injections 1 week previously (Chart 1, Step I). After a time interval of 4 days during which leukemia cells multiplied (Chart 1, Step II), groups of approximately 15 mice were given antileukemic and immunosuppressive therapy (Chart 1, Step III) consisting of 400 R TBR and 5 mg (approximately 185 mg/kg) CY (generously supplied by Dr. Paul M. Worrall, Mead Johnson Research Laboratories, Evansville, Ind.). These doses of TBR plus CY were insufficient to eliminate all leukemia cells but were sufficiently immunosuppressive to assure engraftment of allogeneic cells (7). Approximately 4 to 6 hr later, 2 x 10^7 bone marrow cells and 10^7 lymph node cells from H-2-mismatched DBA/2 donors were administered i.v. (Chart 1, Step IV). This transplant of immunocompetent cells from unprimed histoincompatible donors...
into immunosuppressed leukemic mice (adoptive immunotherapy, GVL reaction) was the key step of the model. The transplanted DBA/2 cells were used to “seek and destroy” all residual leukemia cells. A time interval of 6 days for the GVL reaction (Chart 1, Step V) was selected because bioassay studies (6, 23) showed that all viable clonogenic leukemia cells (i.e., leukemia cells with sufficient proliferative potential to cause death of the animal) were eliminated from the spleens of leukemic AKR mice following a 6-day GVL reaction. Thus, after 6 days, treatments were initiated to rescue the AKR mice from the potentially lethal concomitant GVH disease induced by the DBA/2 cells (Chart 1, Step VI). The mice were treated with i.p. injections of 0.4 ml CBA anti-DBA/2 serum plus 2 mg (approximately 75 mg/kg) CY. Later that day, the AKR mice were given i.v. injections of 2 x 10^7 bone marrow cells from allogeneic, but H-2-matched, CBA donors in order to provide hematopoietic repopulation (Chart 1, Step VII). After a 7-day delay, 2 groups of mice received a 2nd set of rescue maneuvers consisting of a 2nd injection of 0.4 ml CBA anti-DBA/2 serum and a 2nd transplant of CBA bone marrow cells.

Mice. Inbred 10- to 12-week-old AKR/J (H-2^*) and 10- to 14-week-old DBA/2J (H-2^*) female mice, obtained from The Jackson Laboratory, Bar Harbor, Maine, were used.

Transplanted Lymphocytic Leukemia. A long-passage lymphocytic leukemia (BW5147) was the source of leukemia cells. BW5147 leukemia originated spontaneously in an AKR mouse at The Jackson Laboratory in 1954 (12) and has been passaged s.c. there as a solid tumor through more than 875 transplant generations. It had been maintained as a lymphocytic leukemia by serial i.v. passage in our laboratory through more than 100 transplant generations when these experiments were initiated.

The estimated growth pattern of BW5147 cells (Chart 2) was based upon serial dilution experiments done just prior to this study. The hypotheses upon which Chart 2 is based have been described (6, 20). Known numbers of blast cells in the spleen cell suspensions (rather than total number of spleen cells) were administered, because this procedure has resulted in highly reproducible mortality patterns (20). The concentration of blast cells in the spleen cell suspensions ranged from 61 to 69%. During the period of growth from 10^2 to 10^6 cells, the generation time was estimated to be 14.4 hr. We assumed, as did Skipper et al. (25), that the generation time remained more or less constant during growth from 1 cell to 10^6 cells. The generation time was then presumed to have decreased slightly until death, when the mice held an estimated 10^9 leukemia cells (25). With doses of 10^2 to 10^6 blast cells, no AKR hosts survived. When 10^3 blast cells were administered, 16.7% (5 of 30) of the mice survived. Therefore, assuming a Poisson distribution of the clonogenic leukemia cells in the cell suspensions, approximately 2% of the blast cells were clonogenic. By extrapolation, administration of 1 clonogenic leukemia cell would have caused death with a MST of 18.9 days (Chart 2).

X-irradiation and Chemotherapy. The irradiation conditions have been described (5). Dosimetry was verified with a Victoreen R-meter. CY was given i.p. and cells were inoculated 4 to 6 hr following CY administration.

Cell Suspensions. Bone marrow was flushed from the femora of donor mice with Hanks' balanced salt solution and passed 5 times through stainless steel sieves with a pore size of 70 μm. Lymph node cells were obtained from the mesenteric lymph nodes of donor mice. The lymph nodes were cleansed of fat and connective tissue and processed into a single-cell suspension by methods similar to those described for preparing fetal liver cell suspensions (8). Leukemia cells were collected from the spleens of mice bearing BW5147 for 7 days. Following excision of the spleen, 1 ml of Hanks’ solution was injected into the parenchyma, and the spleen was tamped lightly. Cells in Hanks’ solution poured out of the splenic artery and vein and the suspension was passed twice through a 27-gauge needle. Cells were maintained at 4° while aliquots were counted in a hemocytometer and tested for viability by means of nigrosin dye exclusion.
Immunization of CBA Mice against DBA/2 Tissues. Pilot studies were performed to determine effective treatment regimens that could be used to rescue AKR mice after initiation of a lethal GVH reaction (unpublished studies). Best results were obtained when bone marrow transplants from $H$-2-identical CBA donors were preceded by i.p. injections of CY, 100 mg/kg, or 0.4 ml of an antiserum (a gift of Dr. G. D. Snell, Bar Harbor, Maine) specific for the $H$-2 antigens of the $H$-2-mismatched cells.

For preparation of antiserum for the present experiments, CBA mice were hyperimmunized with cells from DBA/2 mice. The immunization schedule was similar to that used by Snell (personal communication) in the preparation of his antiserum. CBA mice were given 2 i.p. injections of the equivalent of 1/25 thymus gland from DBA/2 donors with an interval of 16 to 21 days between injections. Each mouse was then given 3 to 7 i.p. injections of a brei containing the equivalent of 1/10 thymus gland, 1/10 spleen, and 1/10 submaxillary gland at 4- to 10-day intervals. Blood was collected from the retroorbital venous plexus at 3- to 7-day intervals starting after the 5th immunization. The blood was permitted to clot at room temperature and then centrifuged at 4°; the serum was removed and stored at $-20°$. In vitro cytotoxicity tests of pooled antiserum disclosed >95% kill of DBA/2 spleen cells in a dilution of 1/256. Bone marrow cells from CBA mice immunized to DBA/2 tissues were used as donors of bone marrow cells in Chart 1, Step VII, in certain experiments.

Neuraminidase Treatment of Cells. In other experiments, DBA/2 bone marrow and lymph node cells were incubated with neuraminidase (from Vibrio cholerae, General Biochemicals, Laboratory Park, Chagrin Falls, Ohio) and used to immunize CBA mice or for adoptive immunotherapy. Following incubation with neuraminidase for 15 min at 37°, the DBA/2 cells were placed on ice for 5 to 10 min to stop the enzyme action. Cells were centrifuged at 1000 x $g$ at 4° for 7 min, supernatants were discarded, and the cells were resuspended in fresh Hanks' solution. Three concentrations of neuraminidase were used during incubation: 7.5 units/donor when the immunizing dose was 1/25 thymus gland; 20 units/donor when the immunizing dose was 1/10 thymus gland, spleen, and submaxillary gland; and 2.5 units for each 10⁷ bone marrow or lymph node cells. Cell counts and tests of viability were performed following washing and resuspension of the neuraminidase-treated bone marrow and lymph node cells.

Observation. Mice were observed daily for survival. Following the 1st 100-day period of observation, approximately one-third of surviving mice were killed for tests of chimerism. Daily observation continued for the remaining mice.

Tests of Chimerism. Reciprocal 1-way MLC tests (2) were performed to test for chimerism using pooled spleen cells obtained from 7 experimental mice following the 1st 100-day period of observation. Use of MLC for testing chimerism in rats was reported originally by Elkins (11). The technique for MLC in mice has been described (21).

Histology. Mice that died were autopsied. Histological study of the thymus, spleen, liver, and bone marrow was possible in almost all of the mice, although postmortem autolysis precluded definitive histological diagnosis in a few instances. The tissues were evaluated for histological evidence of leukemia, hematopoietic aplasia, lymphoid aplasia, and GVH disease. Death was ascribed to leukemia when the mice had (a) massive splenomegaly; (b) replacement of normal splenic architecture with diffuse infiltrates of monotonously uniform large lymphoid cells (probably lymphoblasts) with numerous mitotic figures; and (c) large perivascular infiltrates of similar cells in the liver. In those mice with a diagnosis of leukemia, infiltrates of leukemic cells almost always were found in the bone marrow and in approximately one-half of the thymus glands. Death was attributed to hematopoietic aplasia or hypoplasia when the bone marrow and spleen were devoid, or nearly devoid, of granulocytic, erythroid, and megakaryocytic cell lines. Lymphoid aplasia or hypoplasia was diagnosed when the spleen was devoid, or nearly devoid, of lymphocytes. A final diagnosis of GVH disease was made in the absence of leukemia and aplasia when the mice had dermatitis and/or focal or diffuse peripoal hepatic necrosis.

RESULTS

Overall results from the 1st 100 days of the experiments are summarized in Chart 3 and Table 1. The MST for all leukemia control mice (Table 1, Group I; Chart 3, Curve ×) was 8.1 days, and no mouse survived beyond Day 12.

Effect of Neuraminidase-treated DBA/2 cells on GVL Reaction. Bone marrow and lymph node cells from DBA/2 donors were incubated with neuraminidase prior to their administration to initiate the GVL reaction (Table 1, Group 2; Chart 3, Curve ●). For rescue, all mice received antiserum from CBA mice immunized with neuraminidase-treated DBA/2 cells, and 13 of these received bone marrow cells from the immune CBA donors. The remaining 15 mice
received bone marrow cells from normal CBA donors. There were no significant intragroup differences and the results have been combined. Most (22 of 28) AKR mice died of BW5147 leukemia. Neuraminidase treatment of the DBA/2 cells appeared to interfere with their GVL capability.

**Rescue from GVH Disease Using Antiserum without CY.** In Table 1, Group 3 (Chart 3, Curve O) the rescue treatment consisted of a single i.p. injection of 0.4 ml CBA anti-DBA/2 serum followed by a bone marrow transplant from CBA donors. CY was not administered as part of the rescue procedure. Although the mortality patterns for Groups 2 and 3, Table 1, were similar (cf. Chart 3, Curves ● and ○), the cause of death was dissimilar. Leukemia was not a significant factor in Group 3. Histological study indicated that 58% of these mice died with aplasia, and at least 21% died with GVH disease. The peak incidence of aplasia occurred between Days 35 and 45. The results suggested that a 2nd transplant of CBA bone marrow might prevent aplasia and that more intense treatment directed against the DBA/2 cells might reduce mortality from GVH disease.

**Rescue from GVH Disease Using Antiserum with CY.** For rescue, mice in Groups 4 and 5, Table 1 (Chart 3, Curves ▲ and △) were treated with CY, antiserum, and CBA cells on Day 10. On Day 17 the mice were given a 2nd injection of antiserum and a 2nd transplant of bone marrow cells from CBA mice. Treatment regimens differed in that CBA donors of bone marrow in Group 4, Table 1 (Chart 3, Curve ▲) had been immunized against DBA/2 tissues. During the 1st 50 days, only 12% (7 of 59) of the mice in these 2 groups died. Assuming that the various treatments caused no significant perturbation in the cytokinetic characteristics of this leukemia (Chart 2), mice in which even 1 residual clonogenic leukemia cell remained should have died well before Day 50. Of the 7 mice that died before Day 50, 4 had histological evidence of leukemia. Thus, apparent cell cure of the transplanted leukemia was accomplished in 88 (52 of 59) to 93% (55 of 59) of the mice in Groups 4 and 5, Table 1. Between Days 50 and 83, an additional 34 mice died. Survival at 100 days for the 2 groups was 31% (18 of 59). Gross and histological examinations of the tissues of mice that died between Days 50 and 83 disclosed that 30 mice died with hematopoietic and lymphoid hypoplasia or aplasia, 1 mouse died with findings typical of GVH disease, and 3 mice died with leukemia. The peak incidence of aplasia was delayed significantly (p < 0.001) to between Days 55 and 70 when compared with Group 3, Table 1.

**Use of MLC's to Test for Chimerism.** Following the initial 100-day period of observation, 7 of the surviving 18 mice in Groups 4 and 5, Table 1, were sacrificed and reciprocal 1-way MLC's were used to test for chimerism (Table 2). Spleen cells from these mice were stimulated to synthesize DNA when cultured with mitomycin C-treated cells from AKR or DBA/2 donors. Conversely, cells from AKR or DBA/2 mice were stimulated when cultured with mitomycin C-treated cells from the experimental mice. Reciprocal nonstimulation was found when cells from the experimental mice were cultured with CBA cells. Thus, AKR mice that had received sequential transplants from DBA/2 donors and from CBA donors approximately 100 days earlier were chimeric, and the predominant cell population in their spleens appeared to be of CBA origin.

**Long-Term Observations.** Of the experimental mice available for long-term observation (Table 3), overall survival at 1 year of age was 73% (11 of 15); 53% (8 of 15) survived beyond 15 months of age, and 3 mice lived more than 21 months. AKR mice surviving more than 15 months repre-
Table 2

<table>
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<tr>
<th>Responder</th>
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<sup>a</sup> Subscript m, mitomycin C treatment of cells; X, pooled spleen cells from 7 AKR experimental mice that were tested for chimerism. Stimulation ratio expressed as X cpm stimulated (s)/X cpm unstimulated (u). cpm of thymidine-<sup>3</sup>H incorporation by responder cells. Numbers shown are mean values of triplicate cultures from 3 separate experiments.

Table 3

<table>
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<th>No. of experimental mice treated and alive at 6 mo. of age&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alive at 12 mo.</th>
<th>Alive at 15 mo.</th>
<th>Alive at 18 mo.</th>
<th>Alive at 21 mo.</th>
<th>Alive at 24&lt;sup&gt;d&lt;/sup&gt; mo.</th>
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<sup>a</sup> Group designations are the same as in Table 1. <sup>b</sup> These mice were the 100-day survivors from Table 1. <sup>c</sup> Seven mice were sacrificed in order to test for chimerism. <sup>d</sup> None of the 5 mice that died between 18 and 24 months of age had gross or histologic evidence of leukemia, aplasia, or GVH disease.

DISCUSSION

Importance of GVL Reaction to Eliminate Transplanted Leukemic Cells. The efficacy of adoptive immunotherapy to treat BW5147 leukemia has been reported in bioassay models (6, 7, 23). In those experiments, clonogenic leukemia cells were eliminated from the spleens of leukemic mice by means of a GVL reaction alone (6) or in combination with chemoradiotherapy (7, 23). Bone marrow and lymph node cells exposed to 3000 R did not produce effective GVL reactions (23).

Im and Simmons (17) reported that the severity of GVH disease was reduced if the effector cells were incubated with neuraminidase prior to administration. In the present experiments, we incubated the DBA/2 bone marrow and lymph node cells with neuraminidase to determine whether (a) pretreated DBA/2 cells were more vulnerable to immunoeлимination (Chart 1, Step VI) because of unmasked antigens (9); (b) GVL reactivity was unchanged; and (c) GVH reactivity was reduced or otherwise altered. The effect of neuraminidase on vulnerability of DBA/2 cells for immunoeлимination and on GVH disease could not be evaluated because of the large number of mice that died of leukemia. The results indicated that the GVL capability of DBA/2 cells was markedly reduced (but not eliminated) as a result of the treatment. Inasmuch as mice in this group received all of the antileukemic chemoradiotherapeutic procedures used in the full model, we consider them to represent a control group that demonstrates the requirement for an unimpaired GVL reaction in order to eliminate residual leukemia cells.

Rescue of Successfully Treated Leukemic Mice from GVH Disease. Termination of an established GVH reaction is a formidable problem (4, 15, 18). In pilot studies (unpublished studies) we found that CY or specific antiserum was most effective in eliminating the cells mediating GVH disease. However, in the present study, specific antiserum without CY was not consistently effective in eliminating DBA/2 cells. Death from GVH disease was reduced markedly when CY was combined with antiserum, although a strict comparison of these rescue protocols is not possible because of a change in the antiserum schedule. Prior immunization of CBA bone marrow donors with DBA/2 cells had no significant effect on the incidence of fatal GVH disease.

Delayed Mortality Associated with Hematopoietic and Lymphoid Aplasia. We observed aplasia in AKR (H-2<sup>b</sup>) mice treated with TBR, CY, and DBA/2 (H-2<sup>2</sup>) cells and rescued with antiserum and CBA (H-2<sup>c</sup>) cells. A similar finding was reported by Barnes and Mole (3), who treated sublethally irradiated CBA (H-2<sup>b</sup>) mice with lymph node cells from C3H (H-2<sup>b</sup>) donors. They found delayed aplasia but did not observe clinical or histologic evidence of GVH disease. The cause for aplasia in their experimental mice is not known (D. W. H. Barnes, personal communication). In both our experiments and in the experiments of Barnes and Mole, aplasia occurred when a transplant of lymph node cells was performed within the H-2<sup>b</sup> genotype, although in our experiments a transplant from DBA/2 (H-2<sup>2</sup>) donors was interposed.

As a consequence of these observations, in subsequent experiments a 2nd transplant of bone marrow from CBA
donors was 1 week after the initial transplant. Many of these mice also developed aplasia and died. However, mortality associated with aplasia was delayed approximately 3 weeks, suggesting that sequential bone marrow transplants from CBA donors might prevent fatal aplasia.

1-Way MLC's to Test for Chimerism. At the conclusion of the 1st 100-day period of observation we wished to determine whether lymphoid cells of surviving experimental mice were of AKR, DBA/2, or CBA origin, or a mixture of cell types. The data indicated that experimental mice were chimeric and that their spleen cells had the in vitro characteristics of the CBA donors. The results reported here confirmed the observation of Elkins (11) that MLC tests may prove useful as a test for chimerism.

Significance of Very Long Survival in AKR Mice. Approximately 90% of untreated virgin female AKR mice develop and die of spontaneous leukemia-lymphoma before they reach 1 year of age, and less than 1% survive to 15 months (22, 26, 27). Following the clinical appearance of AKR spontaneous leukemia-lymphoma, mortality from the disease can be delayed by intensive combination chemotherapy (24). However, chemotherapy does not appear to eliminate the leukemogenic inducing agent because apparent cell cures are followed by reinduction of spontaneous leukemia-lymphoma (26).

In the present studies there were 15 experimental mice available for long-term observation. Following the 1st 100-day period of observation, the mice were approaching 6 months of age and were at risk of development of and death from spontaneous leukemia-lymphoma. The fact that the majority survived beyond 1 year of age was of considerable interest. Although several explanations might be offered for the observed long-term survival of these AKR mice [e.g., the treatment was; in effect, a medical thymectomy (28)], we believe that our results may reflect an antiviral effect of transplanted cells. That is, in addition to GVH and GVL reactions, there may have been a graft-versus-virus reaction.

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