PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 153–158 (1974)

Allogeneic Bone Marrow Chimerism in Germfree Mice
III. Therapy of Leukemic AKR Mice[1] (38061)

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The treatment of hematological malignancies by bone marrow (BM) transplantation has been of continuing interest to investigators. However, a number of problems have prevented an objective evaluation of the procedure. These include graft rejection, graft-versus-host (GVH) disease, and microbial infections. Previous reports from this laboratory have shown (a) that mice with allogeneic BM chimerism survived as long as they remained GF (1); (b) that GF AKR mice with DBA/2 BM cells did not develop spontaneous leukemia (2); and (c) that GF SJL/J mice with C3H bone marrow cells did not develop spontaneous reticulum cell sarcomas (3). Mice of these strains were irradiated and then transplanted at 11 weeks of age with H^{-2} histoincompatible BM cells. The expected neoplastic diseases failed to appear for periods far beyond the age at which the lesions developed in untreated mice. Conventional mice treated in the same manner died of acute GVH disease soon after BM transplantation; and untreated GF irradiated mice died within 10 days.

Several reports have described the use of transplanted spleen or BM cells in the treatment of transplanted murine leukemias (4–9). However, the procedure has been unsatisfactory in mice with spontaneous leukemia. Mathé and Bernard (9) treated the acute "spontaneous" leukemia of AKR mice by transplantation of allogeneic marrow cells into them at advanced stages of leukemia; but a majority of the animals died soon after treatment because of secondary disease. We have previously reported on the prophylactic benefits of transplanted BM cells in hematological malignancies (2, 3); the present report is concerned with the therapeutic aspects of allogeneic chimerism in GF and antibiotic-decontaminated leukemic AKR mice. The preliminary data on GF and on the decontaminated mice offers evidence that transplanted allogeneic (DBA/2) BM cells have therapeutic value for the treatment of spontaneous leukemia in AKR mice.

Methods. Two inbred mouse strains were used: AKR (H^{-2k}) and DBA/2 (H^{-2d}). They have been propagated by brother–sister mating under germfree conditions for more than 20 generations. Mice of each strain were conventionalized by moving them from the GF isolator system to the clean quarters of the conventional animal house where they were further propagated by brother–sister matings.

Groups of conventional AKR mice were decontaminated using a procedure described by van der Waaij (10). Briefly, this involved 12 or more weeks of continuous treatment with nonabsorbed orally administered antibiotics (5 mg Streptomycin, 4 mg Neomycin, 4 mg Bacitracin, and 0.1 mg Amphotericin B/ml drinking water), frequent changes of sterile cages and bedding, and monitoring of the skin and intestines for microbial flora. During this period, the mice were housed in horizontal laminar flow

[1] Supported in part by funds from the U. S. Public Health Service research grant #RR 00294, contract USPHS NO1-CM-23718 and the Marion County Cancer Society.

[2] This investigation was conducted during the tenure of a Damon Runyon Cancer Research Fellowship.

hoods. Leukemic mice were selected from a pool of approximately 60 decontaminated AKR mice.

Spontaneous leukemia in AKR mice is characterized clinically by dyspnea, rough fur, and a hunched (kyphotic) appearance. Because of thick gloves, it was technically inconvenient to palpate lymph nodes in GF and in decontaminated mice, but this served as an additional diagnostic criterion with conventional mice. When AKR mice showed evidence of disease, they were at an advanced stage of leukemia; and if untreated, they rarely survived 10 days. To be most effective, treatment must be given immediately after the appearance of symptoms. When technical difficulties prevented immediate irradiation of leukemic GF mice, they were first administered 5 mg of freshly mixed cyclophosphamide (Cytoxan, Mead-Johnson Co., Evansville, IN) (200 mg/kg body weight) by intraperitoneal injection, and then irradiated 3–4 days later. This served to reduce the primary load of tumor cells and allowed time for transfer of mice into the GF irradiation system.

For whole-body irradiation, decontaminated mice were placed in individual steam-sterilized plastic, wide-mouth bottles which had the bottom removed and replaced with a wire screen and fiberglass filter material. All leukemic mice were irradiated using the procedure and conditions that have been described in an earlier paper (2). GF and decontaminated mice were given 1000 rads, and conventional mice, which are more radiosensitive, were given 850 rads in a single, whole-body exposure at a rate of 39 rads per min. The two doses exceeded the LD_{100} for normal and for leukemic AKR strain mice. The animals were irradiated 3–4 days after cyclophosphamide treatment, or within 2 days of diagnosis if irradiation alone was used.

Twenty-four hours after x-irradiation of the AKR mice, adult donor GF DBA/2 mice were killed by cervical dislocation. The femurs were excised, and their contents were expelled by repeated flushing through the amputated ends by a syringe and needle with sterile Medium 199 solution. The pool of DBA/2 BM cells was refrigerated on crushed ice until transplanted. Each irradiated AKR mouse was injected IV with 10^7 pooled viable BM cells, or the contents of two femurs from mature DBA/2 mice.

The GF chimeric AKR mice were maintained in GF plastic isolators as previously described (11). Decontaminated chimeras were maintained in horizontal laminar flow hoods on sterile Sani-Cell bedding and fed steam-sterilized diet L485 (Teklad) and the antibiotic mixture in water, *ad libitum*. They were examined weekly for microbial status (12).

Control groups of leukemic mice were (a) untreated, (b) given x-ray only, (c) conventional AKR mice with bone marrow from conventional DBA/2 mice, and (d) conventional AKR mice given conventional AKR bone marrow cells.

Results. GF and conventional AKR mice developed spontaneous lymphatic leukemia at an average age of 8 mo (range 3–13 mo), and untreated mice rarely survived 10 days after the appearance of symptoms (13). At autopsy, the primary lesion was an enlarged thymus gland which caused respiratory obstruction. Untreated leukemic mice also showed, in decreasing frequency, swollen spleens, lymph nodes, Peyer's patches, and visceral organs. Some mice had elevated white blood cell counts.

Conventional leukemic AKR mice, irradiated and reconstituted with marrow cells from young adult, non-leukemic AKR mice (syngeneic chimeras), survived for varying periods after transplantation. Table I indicates that of 11 mice, 7 of them died within the critical 60 day observation period and 9 within 90 days. All mice showed the characteristic lesions of untreated lymphatic leukemia, i.e., enlarged thymus, spleen, and lymph nodes.

In contrast, GF leukemic AKR mice, given cyclophosphamide and x-irradiation prior to transplantation of allogeneic DBA/2 BM cells, survived much longer. The mice appeared active within a few days of treatment, although some showed slight weight loss and moderate dyspnea. Eleven of the 12 mice (92%) shown in Table II survived 60 days after treatment. The mouse that died within 60 days did not show autopsy

TABLE I. Transplantation of Conventional Leukemic AKR Mice with AKR Bone Marrow Cells.[a]

Mouse #	Age (mo) at transplant	Status at 150 days after transplant
1	6.9	Dead—39 days—Leukemia
2	6.9	Killed—141 days—Leukemia
3	8.5	Dead—42 days—Leukemia
4	7.1	Dead—49 days—Leukemia
5	7.1	Dead—78 days—Leukemia
6	8.7	Dead—38 days—Leukemia
7	8.3	Killed—125 days—? Leukemia
8	7.6	Dead—75 days—Leukemia
9	7.6	Dead—16 days—Leukemia
10	8.5	Dead—30 days—Leukemia
11	8.5	Dead—23 days—Leukemia

[a] Treatment: 850 rads whole-body x-irradiation + 10^7 syngeneic AKR BM cells.

evidence of AKR leukemia; nor were gross lesions observed in the 4 mice that died after 60 days. At the time of this report, 6 chimeric mice have survived for over 150 days after transplant; a 15-fold increase in their life expectancy at clinical diagnosis. These 6 mice remain under observation at ages exceeding 12 mo.

Decontaminated, allogeneic chimeric mice (AKR with DBA/2 BM) also survived significantly longer than did the syngeneic chimeras (AKR with AKR BM). As shown in Table III, 14 of 17 treated animals (82%) lived beyond 60 days. Of the 3 mice that died within this period, 2 showed no signs of leukemia, while 1, that died 18 days after treatment, had moderate enlargement of the thymus and spleen. Of the 4 mice that died after the 60 day observation period, mouse #1 had histological evidence of leukemia with slightly enlarged thymus and lymph nodes (105 days after treatment), whether the leukemic cells were of host or donor origin was not determined; mouse #3 had gross evidence of leukemia at 142 days after BM transplantation. Mouse, #8 was cannibalized; and mouse #16 had no gross evidence of leukemia. Some of the transplanted mice had marked dyspnea and kyphosis due to the accumu-

TABLE II. Therapy of Germfree Leukemic AKR Mice with DBA/2 Bone Marrow Cells.[a]

Mouse #	Age (mo) at transplant	Status at 150 days after transplant
1	4.9	Dead—48 days—No Leukemia
2	4.9	Dead—62 days—No Leukemia
3	4.9	Dead—131 days—No Leukemia
4	4.9	Alive
5	4.9	Alive
6	6.9	Dead—66 days—No Leukemia
7	6.9	Alive
8	7.3	Dead—139—No Leukemia
9	7.3	Alive
10	7.3	Alive
11	7.6	Dead—147—No Leukemia
12	7.6	Alive

[a] Treatment: 5 mg cyclophosphamide + 1000 rads whole-body x-irradiation + 10^7 allogeneic DBA/2 BM cells.

TABLE III. Therapy of Decontaminated Leukemic AKR Mice with DBA/2 Bone Marrow Cells.[a]

Mouse #	Age (mo) at transplant	Status at 150 days after transplant
1	8.6	Dead—105 days—Leukemia
2	8.6	Alive
3	8.8	Dead—142 days—Leukemia
4	8.8	Dead—34 days—No Leukemia
5	8.8	Alive
6	8.8	Alive
7	9.1	Dead—10 days—No Leukemia
8	9.1	Dead—93 days—Cannibalized
9	9.1	Alive
10	8.9	Alive
11	4.0	Alive
12	4.0	Alive
13	4.0	Alive
14	10.3	Alive
15	10.3	Alive
16	10.6	Dead—61 days—No Leukemia
17	10.8	Dead—18 days—? Leukemia

[a] Treatment: 1000 rads whole-body x-irradiation + 10^7 allogeneic DBA/2 BM cells.

lation of fluid in the thoracic cavity (hydrothorax), which has been associated with radiation-induced kidney damage (2).

Following the transplantation procedure, bacteria were found in fur or fecal samples of some decontaminated mice. However, the bacteria proved transient and disappeared with continued oral antibiotic treatment. At the time of this report, ten of the decontaminated chimeric mice have survived for over 150 days after diagnosis and treatment. Several of these mice are over 15 mo of age, and they remain under observation.

Three irradiated conventional, leukemic AKR mice, inoculated with marrow cells from adult conventional DBA/2 mice, appeared healthy for several days, and then they showed evidence of GVH disease (Table IV). They developed a hunched-up, wasted, and emaciated appearance, scaly dermatitis, and diarrhea. Two of the mice died soon thereafter at 51 and at 55 days post-transplant, while the third was killed after 128 days for tissue specimens. Autopsy examination revealed that the organ components of the lymphoreticular system were small, with no signs of leukemia. The mice showed evidence of pneumonia and hepatitis. Mouse #1, which was killed at 128 days after treatment, showed a less severe GVH reaction than mice #2 and 3. More extensive data on the lethal effects of GVH disease in conventional chimeric mice was reported in earlier papers (2, 3).

All leukemic mice subjected only to the x-irradiation procedure died within 13 days

TABLE IV. Transplantation of Conventional Leukemic AKR Mice with DBA/2 Bone Marrow Cells.[a]

Mouse #	Age (mo) at transplant	Status at 150 days after transplant
1	5.3	Killed—128 days—No Leukemia—GVH*
2	5.3	Dead—51 days—No Leukemia—GVH
3	5.3	Dead—55 days—No Leukemia—GVH

[a] Treatment: 850 rads whole-body x-irradiation + 10^7 allogeneic DBA/2 BM cells.

* GVH = Graft-versus-Host Disease.

with symptoms of wasting and diarrhea. Examinations of leukemic AKR mice at 4 and 5 days after they had received 1000 R x-irradiation did not reveal any leukemic lesions in them.

Discussion. In previous reports from other investigators, immunogenic cells of spleen or of bone marrow origin infused after treatments by drugs or by x-irradiation have induced a limited GVH effect, and destruction of transplanted leukemia cells (4–9). Although the procedure was effective against transplanted leukemic cells, it would be more pertinent if directed against the autochthonous leukemia of AKR mice.

GF technology provides the means whereby the effects of transplanted allogeneic bone marrow cells on hematological malignancies can be evaluated without infectious complications. Previous reports established that under GF circumstances, spontaneous AKR leukemia failed to develop in AKR mice with allogeneic BM chimerism and their longevity was extended significantly. The experiments reported here offer preliminary evidence that the same procedure has therapeutic value for leukemic AKR mice.

Skipper *et al.* (14) evaluated the kinetics of AKR leukemia and arrived at the following conclusions: (a) The average time lapse between the induction of lymphoma cells in the thymus and the appearance of diagnosable lymphoma may be of the order of one month; (b) animals which survive for 60 days or longer after cessation of treatment often represent mice in which all of the original lymphoma cells have been eradicated; and (c) if all but one or several lymphoma cells are eradicated in an animal, or if all are eradicated at the end of therapy and reinduction occurred immediately, then recurrence might be expected 30 days later, and death 16 days after symptoms reappeared, on the average. With respect to the eradication or near-eradication, of lymphoma cells in AKR mice, it was their view that the median survival time after the end of treatment and the number of survivors 60 days or longer after the end of treatment were the most meaningful endpoints.

Because of the continued survival of our chimeric AKR mice, the median survival time cannot be determined at this time. However, using the criteria that leukemic mice that survived 60 days after the end of treatment, i.e., after transplantation, are "cured" of the leukemia, the results show that 25 of the 29 GF and decontaminated mice were actually "cured" by whole-body x-irradiation and transplantation of allogeneic bone marrow cells. In addition three of the four mice that died during the 60-day observation period showed no signs of leukemia at autopsy. The fourth had some enlargement of the thymus and spleen, but without histological examination, the presence of leukemia is not certain.

The term "cured" should be applied cautiously to the transplanted AKR mice, especially since one mouse (#1, Table III) showed histological evidence of leukemia at 105 days after treatment, and another (#3, Table III) showed gross evidence of leukemia after 142 days. It is applied here using the criterion of 60 day survival after treatment, as based on the reported cell kinetics in AKR leukemia (14) and on a comparison with the control syngeneic chimeric mice (Table I). Quantitative evaluation of the efficacy of BM transplantation in "curing" AKR leukemia should be based on large numbers of treated leukemic mice.

The development of lymphatic leukemia in conventional, leukemic AKR mice which had been irradiated and reconstituted with AKR bone marrow cells, was confirmed by histological examination. These mice showed lesions identical to those in untreated leukemic mice. Examinations of leukemic mice 4–5 days after x-irradiation revealed no evidence of leukemia in them. The reappearance of leukemia, approximately 43 days after irradiation and reconstitution with AKR bone marrow cells, may have resulted from the multiplication of leukemia-potential cells in the transplanted syngeneic marrow, or the multiplication of anaplastic cells that were not destroyed by the x-irradiation. The failure of leukemia to recur in GF or in decontaminated allogeneic chimeric mice may have resulted from (a) the introduction of a new regulatory mechanism with the DBA/2 cells; or (b) DBA/2 bone mar-

row cells may lack a specific cell type with leukemogenic potential. DBA/2 mice do not develop leukemia spontaneously.

Although leukemia occurs at the same age and incidence in GF and conventional AKR mice (13), the conventional mouse offers a model closer to the human situation. Conventional AKR mice have been decontaminated, treated, and "cured" of leukemia using the procedure and criteria described above, and this report offers encouraging evidence that transplantation of allogeneic bone marrow cells has therapeutic merit in spontaneous leukemia in AKR mice. As a preliminary study, the extent to which the treatment will extend the life of a leukemic mouse has not yet been determined; nor has the question of whether the treated disease-free animals can be restored to conventional status been determined. Further investigations are needed.

Summary. Conventional AKR mice were decontaminated of bacteria and fungi by means of an antibiotic regimen. When GF and decontaminated mice developed clinical symptoms of leukemia, they were administered 1000 R x-rays (whole-body). Twenty-four hours after irradiation, the mice were injected IV with 10^7 bone marrow cells from GF DBA/2 mice. Eleven of 12 leukemic GF AKR mice treated in this manner survived the 60-day observation period; as did 14 of 17 treated decontaminated leukemic AKR mice. In contrast, of 11 conventional, leukemic AKR mice, which were irradiated and reconstituted with bone marrow cells from nonleukemic AKR mice, 9 died of typical lymphatic leukemia at average 43 days after transplantation. Untreated, leukemic AKR mice died within 10 days after the appearance of leukemic symptoms. Thus, transplanted allogeneic bone marrow cells appear to have therapeutic value for the treatment of spontaneous leukemia in AKR mice.

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Received Dec. 4, 1973. P.S.E.B.M., 1974, Vol. 146.