Allogeneic Bone Marrow Chimerism in Germfree Mice. 1. Prevention of Spontaneous Leukemia in AKR Mice[1] (37657)

MORRIS POLLARD AND ROBERT L. TRUITT

Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana 46556

Immunological mechanisms appear to regulate some neoplastic diseases in animals and man (1–4). Two procedures have attracted considerable attention as possible immunotherapeutic or prophylactic measures: (a) the immunologic enforcement effect resulting from the inoculation of bacteria, such as BCG (5, 6), and (b) the modifying effects derived from inoculation of immunogenic cells of spleen and bone marrow origin (7–10). The first procedure has been used with benefit to tumor-bearing animals; however, the second one involves problems in transplantation biology which require solution. Experimental trials with the latter procedure have shown some encouraging benefits in tumor-bearing animals; however, for the most part, the effects in allogeneic systems have been of short duration because of abortive host-versus-graft rejections, or of lethal graft-versus-host reactions (GVH) (11, 12). The type of GVH disease that occurs after administration of foreign lymphoid cells to either neonatal or older X-irradiated allogeneic recipients is usually acute and fatal. They demonstrate symptoms of wasting (runting), dermatitis, diarrhea, and atrophy of lymphoid tissue. Some amelioration of the GVH reaction has been demonstrated in susceptible animals by treatments with antibiotics (12), which indicates that infection is one of the life-limiting factors in GVH disease.

Investigations in this Laboratory have demonstrated that irradiated germfree (GF) mice, that had been rendered chimeric with H^{-2} incompatible bone marrow cells, would survive over 120 days providing that they remained GF (13, 14). The chimeric mice showed some restoration of the lymphoreticular system, and they manifested specific humoral and cell-mediated immunity. The same experimental protocol has been applied to AKR (15) and DBA/2 mice in this report.

Congenitally transmitted C-type viruses have been observed in the thymic tissues of both GF and conventional mice, representing several genetic strains including AKR. GF AKR mice spontaneously develop lymphatic leukemia in the same pattern as the conventional stock from which they were derived (16): The average incubation period is 8 mo (range 3–13 mo), and they rarely survive for 10 days after the appearance of symptoms. Their primary lesion is a swollen thymus gland (thymoma) in which foci of anaplastic cells expand and involve the entire organ. The enlarged thymus causes respiratory obstruction so that affected mice show severe dyspnea, rough fur, and a hunched (kyphotic) appearance. In addition to the enlarged thymuses, many of the leukemic mice show, in decreasing frequency, swollen spleens, lymph nodes, Peyer's patches, and visceral organs. These enlarged organs are infiltrated with neoplastic cells, many of which are in mitosis. Some mice also show elevated white blood cell counts. While the disease is inevitable in AKR mice, it has been prevented by neonatal thymectomy (17), and deferred by continuous administrations of immunosuppressive agents and interferon (18, 19). DBA/2 mice do not develop leukemia spontaneously.

This report is concerned with the long-term, prophylactic benefits of histoincompatible bone marrow chimerism in GF AKR mice given DBA/2 bone marrow cells. The chimeric AKR mice have not developed thy-

[1] Supported in part by funds from the United Cancer Council, the St. Joseph County and Marion County cancer societies, the Elsa U. Pardee Foundation, and NIH-NCI-C-72-3718.

mic lymphoma, nor other manifestations of lymphatic leukemia, while surviving well beyond the average and extreme incubation periods for the disease.

Methods. Two inbred mouse strains were involved in this investigation: AKR (H^{-2k}) and DBA/2 (H^{-2d}).

Two groups of 20 and 13 GF male and female AKR mice at 11 wk of age were irradiated (whole-body) using a procedure that has been described (14). The conditions for irradiation of both GF and conventional mice were as follows: The source was a 260 kVp therapy X-ray machine operated at 250 kV and 15 mA with a filtration of 1.0 mm Al and 0.25 mm Cu (HLV, 1.05 mm Cu). Mice were irradiated dorsoventrally with a skin–target distance of approximately 50 cm. The animals were placed in a circular polyethylene restrainer separated into 20 wedge-shaped compartments equidistant from the center. Exposure was made at Noon in order to avoid possible diurnal variation in radiosensitivity. Dosimetry, measured in air, was determined in the restrainer under experimental conditions with a 250 R thimble ionization chamber read in a Model 570 Victoreen condenser R-meter. Conventional mice were given 850 rads and GF mice were given 1000 rads in single, whole-body exposure. The two doses exceed the LD_{100} for C3H/He strain mice.

Twenty-four hours later, the 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 syringe and needle with medium 199 solution. The DBA/2 bone marrow cells were refrigerated on crushed ice until inoculated. Each irradiated AKR mouse was injected intravenously with 10^7 pooled viable bone marrow cells, usually the contents of two femurs from mature GF DBA/2 mice.

The chimeric AKR mice were maintained in GF plastic isolators on Sani-cell bedding (20) for periods up to age 15 mo, and examined weekly for microbial status (21). They were fed Teklad diet and water, *ad libitum*. Individual mice were removed periodically for complete histopathological examinations, and their spleen cells were examined by cytotoxicity tests (22) to determine if they were of donor or host type. Each chimeric mouse was killed by ether anesthesia and exsanguinated from the orbital plexus. Representative specimens of all visceral organs and the lymphoreticular tissues were fixed in Bouin's solution, embedded in paraffin, and sections thereof were stained with hematoxylin and eosin.

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

Results. All mice subjected only to the X-irradiation procedure died within 13 days with symptoms of wasting, diarrhea, and pneumonia. Mice irradiated and inoculated within 24 hr with viable syngeneic bone marrow cells survived the acute radiation disease syndrome for varying periods. In this category, among 19 irradiated conventional AKR mice, 9 died within the first month from undetermined causes, and 10 appeared healthy during the next 3 mo. Thereafter, individual mice became sick and died. Two of 3 mice, examined at ages 8 and 10 mo, had the generalized lymphatic leukemia usually observed in untreated AKR mice (Table I). A similar pattern has been observed in a second group of 20 conventional AKR mice which were irradiated and transfused with viable AKR bone marrow cells. Two died within the first month. At age 9 mo, 2 of the mice appeared sick and a third died; and at autopsy the 3 mice had lesions of generalized leukemia. Fifteen mice remain under observation.

Conventional AKR mice were inoculated with bone marrow cells from conventional DBA/2 mice at 24 hr following irradiation. They appeared healthy for several days, then increasing numbers developed the signs and symptoms of GVH reaction: the mice appeared hunched-up with rough fur, and wasted. They developed diarrhea, scaly dermatitis, and walked in mincing fashion on the tips of their toes. They died at average 34 days (range 2–180 days). At autopsy, they showed small thymus glands, lymph nodes, Peyer's patches, and spleens, plus gross and microscopic evidence of pneumonia and hepa-

TABLE I. Effects of Transplanting Conventional AKR Bone Marrow Cells into Irradiated Conventional AKR Mice.

No.	Age (mo)	Sex	Wt	WBC	Hem.	Lesions[b]
19 mice						
D,[a] 1 to 9	4					Negative
D, 10	8					Leukemia
D, 11	10					Negative
K,[a] 12	10	M	23.6	22,800	52	Leukemia
13–19	12	remain				
20 mice						
D, 1	3					Negative
D, 2	3					Negative
K, 3	9		29	1000	47	Leukemia
D, 4	9					Leukemia
K, 5	9	F	33.2	10,450	48	Leukemia
6–20	10	remain				

[a] D = found dead; K = killed.

[b] All mice had cataracts of the optic lens.

titis. The lungs, livers, and the red pulp of the spleens were infiltrated extensively by polymorphonuclear leukocytes. The GVH syndrome observed in these conventional mice with allogeneic bone marrow chimerism was similar to that described previously (11).

In contrast to the lethal effects of allogeneic bone marrow chimerism in conventional mice, the same type of incompatible bone marrow chimerism was relatively innocuous in the GF mice. In the first group of 20 irradiated GF AKR mice with DBA/2 bone marrow cells, 4 died within 120 days after transplantation, for reasons which could not be determined because they were physically degenerated (Table II). After the initial loss of 4 mice, the survivors lived far beyond the average and extreme times in which clinical evidence of leukemia appeared in AKR mice. All of them developed cataracts of the optic lens and some loss of hair. As noted in Table II some of the chimeric mice which appeared sick were killed for examinations. They had developed dyspnea and kyphosis which was subsequently attributed to an accumulation of fluid in the thorax. Except for one mouse with carcinoma of the submaxillary gland, the white blood cell counts were within normal limits for GF mice (av 4580/mm^3, range 1210–7000). The average hematocrit reading was 41% (range 26 to 49). The average body weight of 12 male chimeric mice was 28.3 g (range 24.5–34.4), and of 3 female chimeric mice, 20.7 g (range 18.3–23.2). By cytotoxicity tests, it was established that the spleen cells in chimeric AKR mice were DBA/2 cells. Up to age 15 mo, none of the chimeric AKR mice showed evidence of thymic lymphoma: the thymuses, lymph nodes, and spleens were small. The thymic cortex was clearly defined and repopulated by small lymphoid cells among which were numerous pyknotic nuclei. The medullary area of the thymus appeared incompletely repopulated by cells. The lymph nodes showed distinct cortical follicles of small lymphocytes, devoid of germinal zones; but the paracortical areas were sparsely repopulated. Plasma cells were not observed in the lymph nodes. The Malpighian follicles of the spleen were defined, but without distinct germinal zones. The red pulp of the spleen was reconstituted with megakaryocytes, lymphoid cells, and few polymorphonuclear cells. The Peyer's patches were small aggregates of lymphoid cells, free of germinal zones.

The visceral organs (lungs, liver, kidneys, pancreas, and heart) showed no focal areas of lymphoid cells, which was characteristic of the GF mouse. The lobular pattern of the liver appeared somewhat disorganized. The cecums in all of the GF mice (chimeric and otherwise) were characteristically enlarged and thin-walled with semisolid contents.

TABLE II. Characteristics of Chimeric Germfree AKR Mice with DBA/2 Bone Marrow Cells.

I975, AKR no. 20[a]	Age (mo)	Sex	Wt (g)	WBC/mm³	Hematocrit (%)	Lesions[b]
D, 1	7	—	—	—	—	Degenerated—no gross lesions
D, 2	7	—	—	—	—	Degenerated—no gross lesions
D, 3	7	—	—	—	—	Degenerated—hydrothorax—no gross lesions
D, 4	7	—	—	—	—	Degenerated—no gross lesions
K, 5	10	F	ND	18,400	44	Chronic glomerulonephritis, hydrothorax, ovarian tumor, adenocarcinoma—submaxillary gland
K, 6	11	F	ND	7000	49	Chronic glomerulonephritis, hydrothorax, blast cells in spleen and lymph node
K, 7	13	F	18.3	ND	26	Chronic glomerulonephritis, hydrothorax
K, 8	14	F	ND	6900	42	Chronic glomerulonephritis, hydrothorax
K, 9	14	M	ND	6300	39	Chronic glomerulonephritis
K, 10	14	M	26.4	6300	34	Chronic glomerulonephritis, hydrothorax
K, 11	14	M	25	2300	47	Chronic glomerulonephritis, hydrothorax
K, 12	15	M	30.4	1210	40	Chronic glomerulonephritis, hydrothorax
K, 13	13	F	23.2	3300	30	Chronic glomerulonephritis
K, 14	15	F	20.6	4730	32	Chronic glomerulonephritis, hydrothorax
K, 15	15	M	30.6	4290	36	Chronic glomerulonephritis
K, 16	15	M	24.5	5610	41	Chronic glomerulonephritis
K, 17	15	M	27.2	4510	40	Chronic glomerulonephritis, hydrothorax
K, 18	15	M	34.4	2310	40	Chronic glomerulonephritis, parathyroid tumor

[a] Two were unaccounted for; D = found dead; K = killed; ND = not determined.

[b] All irradiated mice developed cataracts of the optic lens.

There were mitotic cells only in the crypt areas of the intestinal mucosa. The lamina propria contained few cells and the epithelial cover of the mucous membrane was complete.

Two salient lesions were observed in the chimeric, GF AKR mice: They frequently appeared dyspneic and kyphotic because of accumulated clear fluid in the thorax, up to 1.4 ml/mouse. The kidneys in all of the mice were small, and their rough surfaces reflected chronic glomerulonephritis. There were some areas of the cortex in which the tubules were replaced by connective tissue, and some of the glomeruli were relatively acellular and hyaline in appearance. The tissues were free of lymphoid cell aggregations.

Neoplastic lesions were observed in 2 of the GF chimeric mice: One had an adenocarcinoma of the submaxillary gland and an ovarian tumor, and one had a tumor of the parathyroid gland. In one mouse, there were numerous blast cells in the red pulp of the spleen and in the medullary area of one lymph node. This lesion could not be defined.

In the second group of 13 GF AKR mice given allogeneic DBA/2 bone marrow cells, one was dead at 66 days, and at that time, one sick mouse was killed. Both had lesions suggestive of mild GVH disease, or of the spontaneous wasting disease described previously in GF AKR mice (23): they had perianal dermatitis, very small thymus glands, and swollen lymph nodes in which the medullary areas were filled with plasma cells. A third mouse was found dead at age 10 mo, presumably from fighting, and had no evidence of leukemia on gross examination. At age 10 mo, mouse No. 4 was killed because of dyspnea and kyphosis associated with hydrothorax and chronic glomerulonephritis as in the first group of mice (Table II). Thus far, the 9 remaining chimeric mice appear healthy at age 13 mo.

Discussion. The leukemia of AKR mice has been prevented by replacing the lymphoreticular system with bone marrow cells from histoincompatible DBA/2 mice. Some of the early deaths may be due to inadequate cell inoculum because of poor visibility of tail veins through the GF isolator system. The rest of them remained leukemia-negative for periods beyond the age incidence of leukemia in AKR mice. The explanation for this altered manifestation of disease is not clear: (a) irradiation may have deleted a cell type from AKR mice which was responsible for the development of leukemia, or (b) it was replaced by a genetically resistant cell type from donor bone marrow, or (c) the immunological reconstitution of the chimeric AKR mouse was incomplete. Since the thymus is a required "target organ" for murine lymphatic leukemogenesis (17), we wonder if the chimeric AKR mice were actually thymus-deficient. Irradiated AKR mice, reconstituted with AKR bone marrow cells, developed the typical thymic lymphoma syndrome, which would indicate that the leukemogenic factor (cellular or otherwise) was present in the AKR bone marrow inoculum. Perhaps DBA/2 bone marrow cells lacked a specific cell type with leukemogenic potential; or, with DBA/2 cells, a new regulatory mechanism was implanted in the system. As for immunological status of chimeric mice, functioning cell-mediated and humoral types of immunity have been described in them (14, 24).

The lymph nodes, spleens, and Peyer's patches in the chimeric mice were small and appeared to be structurally restored, except for some suggested depletion of thymic dependent areas. The lamina propriae were small, thin, and contained few cells. The white blood cell counts were low. Most of these data are characteristic of the germfree mouse, so the information should be interpreted in relation to the GF not to the conventional mouse.

The development of hydrothorax in the chimeric AKR mice may have resulted from chronic glomerulonephritis, probably induced by the intense irradiation to which they had been subjected. Kidney lesions have been described in mice in association with chronic allogeneic disease (25), exposure to irradiation (26), persistent infection with LCM virus (27), and spontaneously in aging mice (28). The chimeric AKR mice reported here were free of LCM virus, and showed none of the lesions associated with chronic allogeneic disease. The nephrotic syndrome has not been observed in our aging GF mice. It is thus likely that the kidney lesions in our GF

AKR mice resulted from the irradiation effect.

Mice with chronic GVH disease have been reported as having a high incidence of lymphomas (29, 30) which was attributed to an immunological handicap in the affected host. In the above reports, since the recipient mice were not irradiated, they had a functioning immune system. Also, they were inoculated at 6–8 wk of age with spleen cells having weak histocompatible differences, which differed from the protocol involved in our chimeric GF mice. Our chimeric mice were irradiated extensively, then inoculated with bone marrow cells from an H^{-2} incompatible donor, and they manifested none of the lesions (*e.g.*, lymphomas) described in mice with chronic allogeneic disease.

Allogeneic lymphocytes infused after drugs or X-irradiation has induced a limited GVH effect, which destroyed transplanted leukemia cells (7). The results have suggested that adoptive immunotherapy, under carefully controlled conditions, may prove useful as an adjunct to conventional antileukemia therapy. The procedure was effective against transplanted leukemia, but a more significant effect would be directed against the disease that develops spontaneously as in AKR mice. GF mice carrying histoincompatible cells derived from bone marrow have not developed the classical manifestations of GVH, while conventional counterpart mice developed lethal GVH. Under GF circumstances, spontaneous leukemia failed to develop and the longevity of the chimeric mice was extended significantly. Current experiments will determine if the same procedure will "cure" leukemic AKR mice.

Summary. GF AKR (H^{-2k}) mice were administered 1000 R X-rays (whole-body) and 24 hr later they were injected iv with viable bone marrow cells from GF DBA/2 (H^{-2d}) mice. The chimeric GF AKR mice appeared healthy and did not develop spontaneous leukemia up to age 15 mo when the experiment was terminated. Conventional AKR mice with bone marrow cells from conventional DBA/2 mice died of GVH disease. Leukemia appeared spontaneously in conventional AKR mice transplanted with AKR bone marrow cells. Thus, leukemia was prevented in the GF AKR mice with allogeneic chimerism.

1. Prehn, R. T., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **24**, 1018 (1965).

2. Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E. A., and Stockert, E., Cancer Res. **21**, 1281 (1961).

3. Alexander, P., and Hamilton Fairley, G., Brit. Med. Bull. **23**, 86 (1967).

4. Hellstrom, K. E., and Hellstrom, I., *in* "Immunobiology" (R. A. Good and D. W. Fisher, eds.), p. 209. Sinauer Ass. Stamford, CT (1971).

5. Mathe, G., Pouillart, P., and Lapeyraque, F., Brit. J. Cancer **23**, 814 (1969).

6. Zbar, B., Bernstein, I. D., Bartlett, G. L., Hanna, M. G., Jr., and Rapp, H. J., J. Nat. Cancer Inst. **49**, 119 (1972).

7. Barnes, D. W. H., Coop, M. J., Loutit, J. F., and Neal, F. E., Brit. Med. J. **2**, 626 (1956).

8. Fefer, A., "Current Concepts in the Management of Leukemia and Lymphoma," p. 182. Springer-Verlag, Berlin (1961).

9. Boranic, M., and Tonkovic, I., Cancer **31**, 1140 (1971).

10. Bortin, M. M., Rimm, A. A., and Saltzstein, E. C., Science **179**, 811 (1973).

11. Congdon, C. C., and Urso, I. S., Amer. J. Pathol. **33**, 749 (1957).

12. Van Bekkum, D. W., and De Vries, M. J., "Radiation Chimaeras." Academic Press, New York (1967).

13. Jones, J. M., PhD dissertation, Univ. of Notre Dame, Notre Dame, IN 1970.

14. Jones, J. M., Wilson, R., and Bealmear, P. M., Radiat. Res. **45**, 577 (1971).

15. Furth, J., Sibold, H. R., and Rathbone, R. R., Amer. J. Cancer **19**, 521 (1933).

16. Pollard, M., Kajima, M., and Teah, B. A., Proc. Soc. Exp. Biol. Med. **120**, 72 (1965).

17. McEndy, D. P., Boon, M. C., and Furth, J., Cancer Res. **4**, 377 (1944).

18. Pollard, M., and Sharon, N., Proc. Soc. Exp. Biol. Med. **137**, 1494 (1971).

19. Gresser, I., Coppy, J., and Bourali, C., J. Nat. Cancer Inst. **43**, 1083 (1969).

20. Trexler, P. C., Ann. NY Acad. Sci. **78**, 29 (1959).

21. Wagner, M., Ann. NY Acad. Sci. **78**, 89 (1957).

22. Boyse, E. A., Old, L. J., and Chouroulinkov, I,. *in* "Methods in Medical Research" (H. N. Eisen, ed.), p. 39. Year Book Med. Pub., Chicago (1964).

23. Pollard, M., Nature (London) **222**, 92 (1969).

24. Loughman, B. E., PhD dissertation, Univ. of

Notre Dame, Notre Dame, IN 1972.

25. Lewis, R. M., Armstrong, M. Y. K., Andre-Schwartz, J., Muftuoglu, A., Beldotti, L., and Schwartz, R. S., J. Exp. Med. **128**, 681 (1968).

26. Cole, L. J., Nowell, P. C., and Arnold, J. S., Radiat. Res. **12**, 173 (1960).

27. Kajima, M., and Pollard, M., Amer. J. Pathol. **61**, 117 (1970).

28. Kirschbaum, A., Proc. Soc. Exp. Biol. Med. **64**, 71 (1944).

29. Armstrong, M. Y. K., Gleichmann, E., Gleichmann, H., Beldotti, L., Andre-Schwartz, L., and Schwartz, R., J. Exp. Med. **132**, 417 (1970).

30. Walford, R. L., Science **152**, 78 (1966).

Received July 10, 1973. P.S.E.B.M., 1973, Vol. 144.