PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 403-407 (1976)

Relationship between Age and Thymic Function in the Development of Leukemia in AKR Mice (39406)

RANDY J. GERSHWIN, M. ERIC GERSHWIN,[1] ALFRED D. STEINBERG, AFTAB AHMED, AND TAKENORI OCHIAI

Department of Medicine, University of California School of Medicine, Davis, California 95616; the Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health; and the National Naval Medical Research Institute, Bethesda, Maryland 20014

AKR mice develop spontaneously lymphocytic leukemia and have served as models for study of murine leukemia virus (1-3). Although the virus is present in the thymus before birth, disease is not manifest until the mice are at least 6 months old (4, 5). Beginning at that age, frank lymphoid infiltrates develop in most parenchymal organs, often with a thymoma and marked lymphocytosis (1-5).

The immunopathologic mechanism responsible for this latent period is largely unknown but several key observations have been described. Thymectomy before 8 weeks of life markedly reduced the incidence of leukemia (1, 6). Leukemogenesis can be accelerated in these thymectomized animals, with the typical latency period, by transplantation of young thymic grafts (6). Moreover, young thymectomized mice transplanted with a 6-7 month syngeneic thymic graft develop leukemia without a latency period. In addition, the incidence of leukemia is reduced in AKR-C3H chimeras (7). These observations have led investigators to propose that the latency period is due to the presence of thymic regulatory or suppressor cell activity in young mice (7, 8). We have herein investigated the role of the thymus and its relation to the age of the animal in the development of leukemia in AKR mice. In addition, we have tested the hypothesis that thymic derived cells, capable of inhibiting neoplastic transformation and progression, are present in young mice and are lost with age by repetitive transplantation of either young (1 month) or old (6 month) thymic grafts.

Materials and methods. Mice. AKR/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were housed 10 per cage and fed standard lab chow and water *ad libitum*. Other strains of mice used in this experiment: A/LN, BALB/C, NZB, and DBA/2 were obtained from the Rodent and Rabbit Production Unit, National Institutes of Health, Bethesda, Md. Only male mice were used in this study.

Survival studies. Two-month-old AKR mice were randomly divided into two groups. One (consisting of 25 mice) was grafted with a 1-month-old AKR thymus every fortnight. The second (20 mice) was untreated and served as a control group.

Additional 2-month-old AKR mice were divided into three groups: (i) Thirty mice were thymectomized and 10 days later given a 6-month AKR thymus graft. (ii) Thirty mice were sham-thymectomized and 10 days later given a 6-month AKR thymus graft. (iii) Twenty-five mice were sham-thymectomized and not grafted.

Sixty-nine 6-month-old AKR mice were ramdomized into three groups of equal size. The first group was untreated. The second received repetitive transplants every fortnight of 1-month-old AKR thymic grafts. A third group received every fortnight thymic grafts from 6-month-old AKR mice.

Thymic transplants. Donor mice were sacrificed by cervical dislocation and thymectomy was performed. The thymus from a 1-month animal was divided into two lobes so that each recipient received one lobe. The thymus from 6-month animals was used intact. In all cases the contiguous lymph nodes and fatty tissue were carefully dissected from thymic graft and was implanted subcutaneously in the axilla of recipient mice; the wound was closed with a stainless steel clip. Mice receiving repetitive transplants were grafted initially in the axilla and thence sub-

[1] Author to whom reprint requests should be addressed: TB 171, University of California at Davis, Davis, Calif. 95616.

sequently in the flanks and dorsum. The survival of the transplants were confirmed by palpation of the graft during the survival phase and by histologic examination when moribund. Additionally, the survival of the transplants was confirmed, in mice sacrificed, for mitogen studies, by light microscopy with further identification of thymocytes using anti-theta sera (9). All mice were followed for survival and autopsied when moribund. Survival data between groups was prepared using the Kolmogorov–Smirnov nonparametric test (10).

Transplantation of AKR thymomas. Spontaneously occurring thymomas were removed at autopsy (performed on moribund animals) and gently minced into tissue fragments 2 × 2 mm in Hanks Balanced Salt Solution (Grand Island Biologic Co., Grand Island, N.Y.). The fragments were placed subcutaneously in the flank of groups of eight AKR mice of each of the following ages: 4, 8, 12, 20, 28, and 36 weeks. Additional recipients were eight 8-week-old AKR mice thymectomized 2 weeks earlier. The size of the graft and survival of recipients was followed daily. Similar grafting experiments were performed using 4- and 24-week NZB, BALB/c, DBA/2, and A/LN mice.

Mitogen studies. Groups (four mice per group) of 6-month and 10-month AKR mice repetitively transplanted from 8 weeks of age with thymic grafts from 4-week-old AKR mice were sacrificed by cervical dislocation. Groups (four to eight mice per group) of 2-, 6-, and 10-month-old untreated AKR mice served as controls. The spleen, mesenteric, and femoral lymph nodes were removed and placed in RPMI 1640 media (Grand Island Biologic Co.). Single-cell suspensions of spleen and lymph nodes were prepared by passing the tissue through sterile 22-gauge nylon mesh, teasing the resultant fragments and shearing clumps through a 25-gauge needle. Viability was confirmed by trypan blue exclusion. Each animal was studied individually. The cell suspensions were cultured in microtiter plates as previously described (11). To sextuplicate cultures, from each animal, were added 100 μl of each mitogen: phytohemagglutinin-P (PHA-P), 0.1% final concentration (Difco Labs, Detroit, Mich.); Concanavalin A (Con A), 0.25 μg/culture (Calbiochem, San Diego, Calif.); *Escherichia coli* lipopolysaccharide (LPS), 50 μg/culture (Difco Labs); and polyriboinosinic acid, polyribocytidylic acid [poly(I)·poly(C)] 50 μg/culture (P-L Biochemicals, Milwaukee, Wis.); or RPMI 1640 alone. Cultures were incubated for 72 hr at 37° in a 5% carbon dioxide, 80% oxygen, 15% nitrogen-humidified atmosphere. Eighteen hours before harvest, each received 1 μCi of [methyl-^{3}H]thymidine ([^{3}H]Tdr) (1.9 Ci/mmole, Schwartz-Mann, Orangeburg, N.Y.) in 20 μl of media. Cultures were harvested with

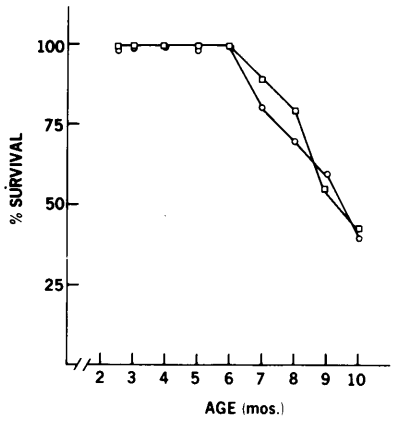

FIG. 1. Percentage survival plotted against age in untreated control mice (□—□) and mice transplanted every fortnight with 4-week AKR thymus (○—○) beginning at age 8 weeks.

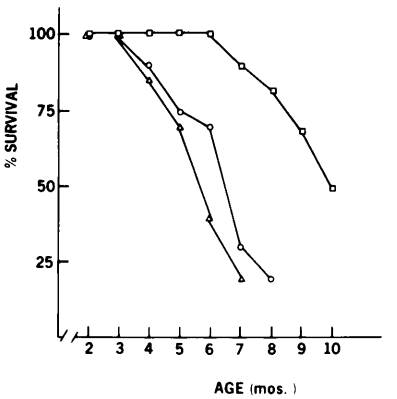

FIG. 2. Percentage survival plotted against age in (a) mice undergoing sham thymectomy at 8 weeks of age (□—□); (b) mice thymectomized at 8 weeks of age transplanted 10 days later with a 6-month thymic graft (△—△); and (c) 8-week-old mice undergoing a sham thymectomy and transplanted 10 days later with a 6-month thymic graft (○—○).

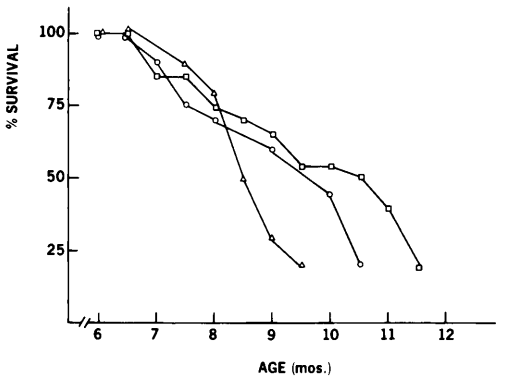

FIG. 3. Percentage survival plotted against age in (a) 6-month, unmanipulated, AKR mice (□—□); (b) 6-month mice grafted every fortnight with 4-week syngeneic thymus (○—○); (c) 6-month mice grafted every fortnight with 6-month thymus (△—△).

an automated multiple sample harvester.

Results. There was no evidence to suggest an inherent resistance of young AKR mice or young AKR thymic cells to development of leukemia. AKR mice, 8 weeks old at study entrance, repetitively grafted with 4-week grafts had identical survival to untreated controls (Fig. 1). Mortality was accelerated by grafting 8-week-old AKR mice with 6-month syngenic thymic grafts (Fig. 2). However, the prevalance of leukemia was similar in the 8-week-old AKR mice receiving a single 6-month graft, whether or not they had been thymectomized (Fig. 2). Repetitive grafting of 4-week thymic tissue into mice 6 months of age at study entrance did not prolong survival (Fig. 3). However, mice 6 months old at study entrance, receiving repetitive grafting of 6-month thymic tissue, developed leukemia earlier (Fig. 3). The 50% mortality for this group was significantly shorter ($P < 0.01$) than the control group.

The AKR (thy 1.1) thymus transplants survived in the recipients. This was confirmed by palpation, histology, and use of anti-thy 1.1 sera. The growth rate and survival of transplanted thymomas in AKR mice was unrelated to age (Table I). As might be expected, AKR thymomas do not survive in θ-C3H (thy 1.2) strains of mice (NZB, BALB/c, DBA/2, or A/LN). It is noteworthy that no growth was observed in A/LN mice as they, unlike the other strains, have the theta-AKR (thy 1.1) allele, and thy 1.1 has been observed on AKR lymphoma cells (12).

There was progressive loss of PHA-P and Con-A responsiveness of spleen cells as AKR mice aged (Fig. 4). A lesser relation in responses to pokeweed mitogen and LPS (Primarily B cell mitogens) was observed with age (Fig. 4). Lymph node cells which respond minimally to T cell mitogens also showed a decrease with age (Fig. 5).

Discussion. It is generally accepted that thymectomy of young AKR mice markedly reduces the incidence of leukemia. However, several critical findings have suggested that, in addition to harboring the murine leukemia virus, the AKR thymus may play a role in the long latency period before disease becomes manifest (7, 8). Treatment of AKR mice with antilymphocyte serum accelerated leukemogenesis (13). Further, leukemia is retarded in allogeneic bone marrow chimeras as well as in tetraparental ovum fusion-derived AKR chimeras (7, 8, 14, 15).

The present study strongly indicates little or no significant active role for the AKR thymus in retarding leukemogenesis. Subcutaneous thymoma grafts of AKR origin grew equally well in AKR recipients of all ages. Further, repetitive transplantation of young syngeneic thymic tissue into 8-week or 6-month mice, did not alter survival. In

TABLE I. RESPONSE TO SUBCUTANEOUS TRANSPLANT OF AKR THYMOMA.[a]

Recipients	Age[b]	Growth[c]	Survival[d]
AKR/J	4	15.9	39.4
AKR/J*	8	15.3	35.7
AKR/J	8	13.8	41.2
AKR/J	12	16.7	36.8
AKR/J	20	15.1	44.2
AKR/J	28	17.4	32.1
AKR/J	36	14.9	27.4
NZB	4	0	>90
NZB	24	0	>90
BALB/c	4	0	>90
BALB/c	24	0	>90
DBA/2	4	0	>90
DBA/2	24	0	>90
A/LN	4	0	>90
A/LN	24	0	>90

[a] Groups of four to eight mice.
[b] Age in weeks.
[c] Mean time in days to reach palpable tumor mass of 1 × 1 cm^3.
[d] Mean survival in days.
* Thymectomized at age 6-weeks.

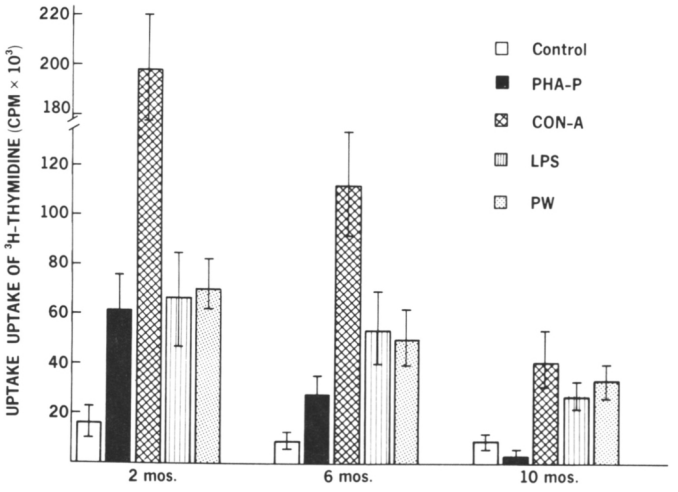

FIG. 4. Incorporation of [³H]thymidine by spleen cells from AKR mice 2, 6, or 10 months of age.

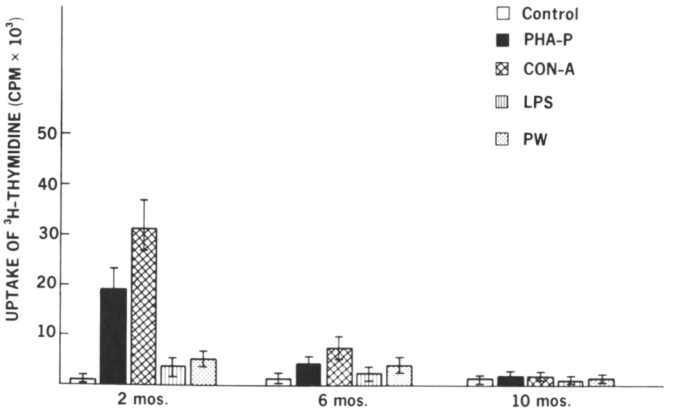

FIG. 5. Incorporation of [³H]thymidine by lymph node cells from AKR mice 2, 6, or 10 months of age.

fact, repetitive grafting of 6-month thymus into mice, beginning at age 6 months, accelerated disease. This acceleration of leukemogenesis may be secondary to the larger potential source of premalignant (thymus) tissue. These results are consistent with observations that spontaneous antilymphoma cytotoxic reactions of preleukemic AKR mice are not T-cell dependent (16).

Immunotherapy of leukemia in AKR mice has been largely disappointing. Although antibody to virus exists it occurs primarily as antigen-antibody complex (17, 18). Cell mediated immunity to virus has not been demonstrated. Interferon administration retards leukemogenesis (19). Similarly, pyran-2-succinic anhydride-4,5-dicarboxytetrahydro-6-methyl-anhydride polymer (NSC-46015), an interferon inducer, retards disease (13). Finally, lethal irradiation of leukemic AKR mice, followed by restoration with bone marrow cells from germ-free DBA mice prevents leukemia (14). The present study militates against use of syngeneic tissue transplants and in favor of further understanding the host-viral relationship before immunotherapeutic manipulation.

The mitogen studies indicate a progressive loss of response to the T cell stimulators Con-A and PHA-P. This is similar to studies in patients with chronic lymphocytic leukemia and may reflect either a loss of T cells or a dilution of T cells by either a null cell or B cell population (20–22). It is of particular interest that a decrease in responsiveness of

spleen and lymph node cells to Con-A and PHA-P preceded frank leukemia. Further, mice receiving thymic grafts had identical mitogen responsiveness as unmanipulated controls. Neoplastic transformation appears to occur, therefore, more as a consequence of the virus natural history than of the immunologic condition of the AKR host.

Summary. The characteristic latency period of 6 months was noted for the development of leukemia of AKR mice. Growth of transplanted syngeneic thymomas occurred equally well in 4-week- as 28-week-old recipient AKR mice. Repetitive grafting of thymic tissue from 4-week donors into AKR mice 8 weeks or 6 months old at initiation of study failed to retard leukemogenesis. This result is contrary to the hypothesis that the latent period for leukemia in AKR mice is produced by thymic suppressor cells in young AKR mice. Further, transplantation of 6-month thymic tissue into either 8-week or 6-month recipients accelerated disease. There was a progressive loss of responsiveness to phytohemagglutinin and concanavalin A in AKR mice with age. The responsiveness to mitogens during frank leukemia was similar to that described in patients with chronic lymphocytic leukemia.

1. Law, L. W., and Miller, J. H., J. Nat. Cancer Inst. **11,** 425 (1950).
2. Law, L. W., and Potter, M., Proc. Nat. Acad. Sci. USA **42,** 160 (1956).
3. Furth, J., Kunii, A., Ioachim, H., *et al.*, "The Thymus: Experimental and Clinical Studies" (G. E. W. Wolstenholme and R. Porter, eds), p. 288. Little, Brown, and Co., Boston. (1966).
4. Gross, L., Proc., Soc. Exp. Biol. Med. **78,** 342 (1951).
5. Metcalf, D., "The Thymus, Recent Results in Cancer Research," Vol. 5. Springer-Verlag, New York (1966).
6. Nagaya, H., J. Immunol. **111,** 1048 (1973).
7. Barnes, R. D., Tuffrey, M., and Kingman, J., Clin. Exp. Immunol. **12,** 541 (1972).
8. Barnes, R. D., Tuffrey, M., and Ford, C. E., Nature New Biol. **244,** 282 (1973).
9. Gershwin, M. E., Chused, T. M., and Steinberg, A. D., Transplantation **18,** 377 (1974).
10. Sieger, S., "Nonparametric Statistics for the Behavioral Sciences." McGraw-Hill, New York (1966).
11. Strong, D. M., Ahmed, A., Thurman, G. B., and Sell, K. W., J. Immunol. Meth. **2,** 279 (1973).
12. Grey, H. M., Colon, S., Campbell, P., *et al.*, J. Immunol. **109,** 776 (1972).
13. Hirsh, M. S., Black, P. H., Wood, M. D., and Monaco, A. P., J. Immunol. **111,** 91 (1973).
14. Truitt, R. L., Pollard, M., and Srivastava, K. K., Proc. Soc. Exp. Biol. Med. **146,** 153 (1974).
15. Pollard, M., and Truitt, R. L., Proc. Soc. Exp. Biol. Med. **144,** 659 (1973).
16. Gomard, E., Leclerc, J. C., and Levy, J. P., Nature (London) **250,** 671 (1974).
17. Kassel, R. L., Old, L. J., Carswell, E. A., *et al.*, J. Exp. Med. **138,** 925 (1973).
18. Oldstone, M. B., Aoki, T., and Dixon, F. J., Proc. Nat. Acad. Sci. **69,** 134 (1972).
19. Gresser, I., Coopey, J., and Bourali, C., J. Nat. Cancer Inst. **43,** 1083 (1969).
20. Nagaya, J., J. Immunol. **111,** 1052 (1973).
21. Fernandez, L. A., MacSween, J. M., and Langley, G. R., Immunology **28,** 231 (1975).
22. Smith, J. L., Cowling, D. C., and Barker, C. R., Lancet **1,** 229 (1972).

Received December 9, 1975. P.S.E.B.M. 1976, Vol. 152.