

Effect of Sex Hormones on Susceptibility to Viral Transmission of Myeloid Leukemia in the Mouse¹

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Summary

The effects of sex hormones on the incidence and latency of myeloid leukemia induced by ultracentrifugates were studied in RF mice. Mice treated with estradiol had a lower incidence and a longer latency of myeloid leukemia than mice treated with the injection vehicle (sesame oil) or with testosterone. These results support earlier findings that male mice of the RF strain are more susceptible to leukemia induction by cell-free filtrates or by radiation than are females.

Introduction

The influence of the gonads on leukemogenesis in the mouse varies from strain to strain (11) and with the hematologic type of leukemia (21). In mice of certain strains prone to high incidence of lymphoid leukemia, the presence of the testes has been shown to inhibit spontaneous development of the disease and ovariectomy to have no effect (14, 16). In other strains, the presence of the ovaries has been reported to account for the higher incidence of lymphomas in females (15). Radiation-induced lymphomas are more common among females than males in certain strains of mice (6, 10, 20) but not in others (3). In some of these strains, estrogens enhance the leukemogenic action of x-rays and androgens inhibit it (5, 7, 10).

Although spontaneous myeloid leukemia occurs infrequently in most strains of mice (2), its incidence approximates 3-5% in mice of the RF strain (1, 19). Male RF mice show an incidence approaching 40% and females 20% in response to 300 r of whole-body x-radiation early in adult life (19, 22). A sex-dependent difference in susceptibility to induction of myeloid leukemia also occurs in newborn RF mice treated with cell-free filtrates, the incidence being greater in the males than in females (8).

In view of the evidence implicating sex hormones in the pathogenesis of spontaneous (11), radiation-induced (22), and filtrate-induced (8) leukemias, this study was undertaken to explore the effects of sex hormones on viral transmission of myeloid leukemia in the RF mouse.

Materials and Methods

HANDLING OF MICE. The animals used were random-bred mice of the RF strain produced in our laboratory. Four-week-old

intact and gonadectomized males and females were injected i.v. with ultracentrifugates prepared from mice bearing passaged myeloid leukemia. Mice of each sex were randomized, caged in groups of 9, and given Purina laboratory chow and fresh drinking water *ad libitum*. Mouse cages were changed semi-weekly, and chlorinated drinking water was changed 3 times each week.

PREPARATION OF LEUKEMOGENIC ULTRACENTRIFUGATES. Mice with splenomegaly, pallor of ears and tail, and increased numbers of promyelocytes and myelocytes in the blood were killed by cervical separation or Nembutal anesthesia and their spleens removed aseptically. Parts of the spleen, kidney, liver, and bone marrow were fixed in Zenker's solution for microscopic confirmation of the gross diagnosis of myeloid leukemia. The remaining spleen tissue, which weighed about 1.0-1.5 gm, was finely minced with sterile surgical scissors and suspended in sterile Tyrode's solution. The resulting spleen cell suspensions, containing 100-150 × 10⁶ nucleated cells/ml as determined by counting in a conventional hemocytometer, were centrifuged in a Servall RC-2 refrigerated centrifuge at 0-5°C. The original cell suspension was centrifuged at 1800 × *g* for 20 min (Chart 1). The resulting supernatant fluid was removed by pipet and centrifuged at 8000 × *g* for 10 min, and the resulting supernatant was similarly centrifuged at 8000 × *g* for 10 min. The resulting supernatant fluid was then removed by pipet and centrifuged at 30,000 × *g* for 20 min. The pelleted sediment (*Sp4*) from the 30,000 × *g* centrifugation was suspended in cold Tyrode's solution and injected into the test mice; the supernatant fluid (*Ss4*) from the 30,000 × *g* centrifugation was used in 8 passages, and the pellet (*Sp3*) from the second 8000 × *g* centrifugation was used in 2 passages. In every passage, the material from a given donor was injected via the tail vein into 3 recipients of each treatment group in the volume of 0.5 ml/recipient.

GONADECTOMY OF RECIPIENTS. Ovariectomy was performed aseptically on 3-week-old weanling females under Nembutal anesthesia. Anesthetized animals were shaved on their backs from the base of the tail to the thoracic region and swabbed with iodine and 70% ethyl alcohol. A 1-cm-long middorsal incision was made through the skin, and the ovaries were removed through dorsolateral incisions 2-3 mm long in the peritoneum adjacent to the ovaries. After removal of both ovaries, hemostasis of the blood vessels occurred spontaneously, and the skin was closed with surgical silk.

Orchidectomy was performed aseptically on 3-week-old weanling males under Nembutal anesthesia. After the scrotum had been shaved and swabbed with iodine and alcohol, an incision 0.5 cm long was made at the midline and the testes were removed through the incision with sterile forceps. Section of

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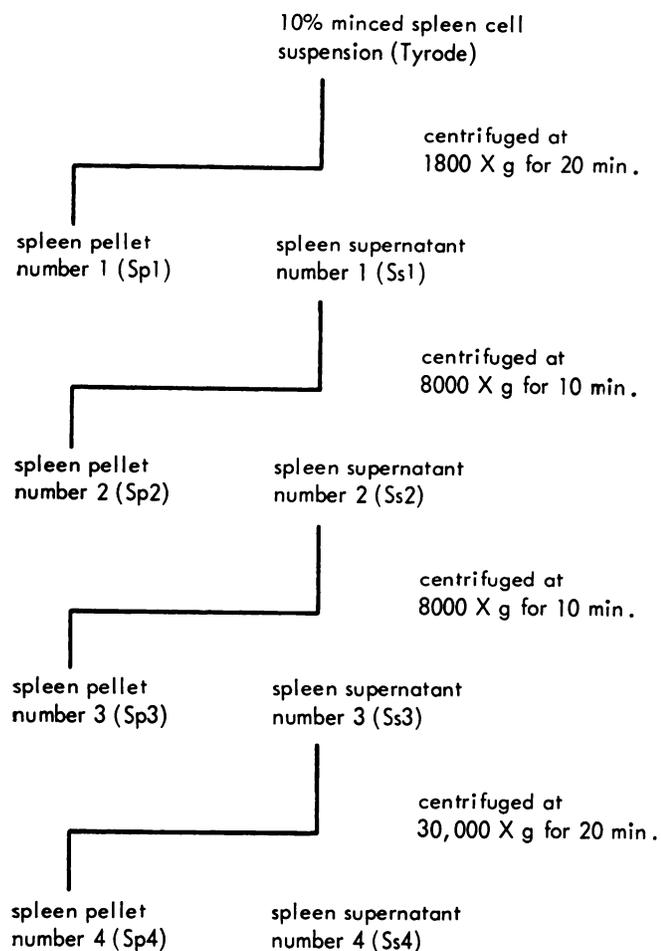


CHART 1. Schematic diagram of procedure for preparing the ultracentrifugates from spleens of mice with myeloid leukemia.

the vas deferens was made, care being taken to remove all testicular tissue, after which the incision was closed with surgical silk.

ADMINISTRATION OF SEX HORMONES. In the groups given estrogen (Table 1), i.m. injections of 0.33 mg of estradiol benzoate (Progynon benzoate, Schering Corporation, Bloomfield, New Jersey) in 0.2 cc of sesame oil were administered in the thigh muscle weekly, the 1st injection on the day of inoculation of leukemic ultracentrifugate, and continued for 90 days. In other groups, testosterone propionate (Oreton propionate, Schering Corporation, Bloomfield, New Jersey) was injected in the thigh muscle weekly, 1 mg in 0.2 cc of sesame oil. In other groups, the hormone vehicle (sesame oil) was injected weekly into the thigh muscle in the same volume as the hormones.

DETERMINATION OF INCIDENCE OF MYELOID LEUKEMIA. Mice dying within 90 days after injection of ultracentrifugates were considered positive for myeloid leukemia if their necropsy revealed splenomegaly, hepatomegaly, and other signs characteristic of myeloid leukemia (20). Specimens of the blood-forming organs were removed from animals examined within a few hours after death and were fixed in Zenker's solution for preparation of histologic slides. The gross diagnosis was confirmed by microscopic examination of the histologic slides. Mice found

moribund with clinical signs of leukemia were killed, since such animals have been found usually to die within 6-8 hr.

The majority of the leukemias displayed a predominance of promyelocytes and myeloblasts. Splenomegaly and hepatomegaly were constant features of the disease. The thymus and lymph nodes were usually of normal size, but infiltration of the lymphatic tissues by myeloid elements was observed in several of the mice examined with characteristics of chloroleukemia.

Results

EFFECTS OF ULTRACENTRIFUGATES ON MYELOID LEUKEMIA INCIDENCE IN INTACT MICE. Myeloid leukemia has not been observed in RF mice during the 1st 4 months of life except after inoculation of viral filtrates or ultracentrifugates (22, 8). After injection of ultracentrifugate alone, the incidence reached 12.8% in females and 16.1% in males within 90 days (Table 1). Injection of vehicle (sesame oil) in addition to the ultracentrifugate did not significantly change the incidence or latency in intact females (12.5%) or males (20.5%).

EFFECTS OF SEX. The incidence of myeloid leukemia was higher in all groups of males, except for the estrogen-treated nongonadectomized group, than in comparable groups of females (Table 1). However, this sex difference could not be established as statistically significant by comparison of individual groups numbering 96 mice/group. When all groups of inoculated males were combined (97/576 = 16.8%) and compared with comparable combined groups of females (68/576 = 11.8%), the sex difference was significant ($P < 0.01$).

The leukemia in female mice usually had a longer latency than in comparably treated males (Table 1; Charts 2, 3). The mean latency of combined female treated groups (67 days) was significantly longer ($P < 0.05$) than that of combined male groups (63 days).

EFFECTS OF GONADECCTOMY. The incidence of induced leukemia in gonadectomized males (20.8%) was the same as that in intact males (20.8%) of the vehicle-treated groups (Table 1). Statistical comparison of the combined data for all hormone- and vehicle-treated males failed to show a significant difference between the intact (44/288 = 15.3%) and the gonadectomized (53/288 = 18.4%) animals.

Comparison between gonadectomized and intact females revealed similar incidences of induced leukemia in vehicle- and testosterone-treated groups (Table 1). The incidence in estrogen-treated mice was lower in the gonadectomized group (5.2%) than in the intact group (11.5%), but this difference was not statistically significant ($P > 0.05$).

Gonadectomy did not alter the latency of leukemia in either sex (Table 1).

EFFECTS OF VEHICLE. The incidence and latency of leukemia in vehicle-injected mice were comparable to those in mice of the same age and sex which received neither hormone nor vehicle (Table 1).

EFFECTS OF TESTOSTERONE. Testosterone treatment resulted in little or no change in incidence in females or males (Table 1). Statistical comparison of the incidence in all control (vehicle-treated) mice (66/384 = 17.2%) with that in all testosterone-treated mice (58/384 = 15.1%) showed no significant difference between the 2 groups.

TABLE 1

INCIDENCE AND LATENCY OF MYELOID LEUKEMIA DURING THE 1ST 90 DAYS AFTER INJECTION IN RELATION TO SEX AND TREATMENT

TREATMENT		FEMALES						MALES								
Ultracentrifugate	Hormone	Intact			Gonadectomized			Intact			Gonadectomized					
		Inc. ^a		Lat. ^b (days)	Inc.		Lat. (days)	Inc.		Lat. (days)	Inc.		Lat. (days)			
		No.	%		No.	%		No.	%		No.	%				
None	None	0/1024 ^c				0/118 ^c				0/614 ^c				0/103 ^c		
Ultracentrifugate	None	20/156	12.8	64					19/118	16.1	65					
Ultracentrifugate	Vehicle	12/96	12.5	67	14/96	14.6	63	20/96	20.8	64	20/96	20.8	61			
Ultracentrifugate	Testosterone	12/96	12.5	66	14/96	14.6	69	14/96	14.6	56	18/96	18.8	61			
Ultracentrifugate	Estrogen	11/96	11.5	70	5/96	5.2	68	10/96	10.4	66	15/96	15.6	69			
	Total for vehicle, testosterone, estrogen	35/288	12.1	68	33/288	11.5	67	44/288	15.3	62	53/288	18.4	64			

^a Incidence: number of mice developing the disease within 90 days over the number injected.

^b Latency: mean number of days after ultracentrifugate injection until development of the disease.

^c Data from Upton *et al.* (22) and Jenkins and Upton (8).

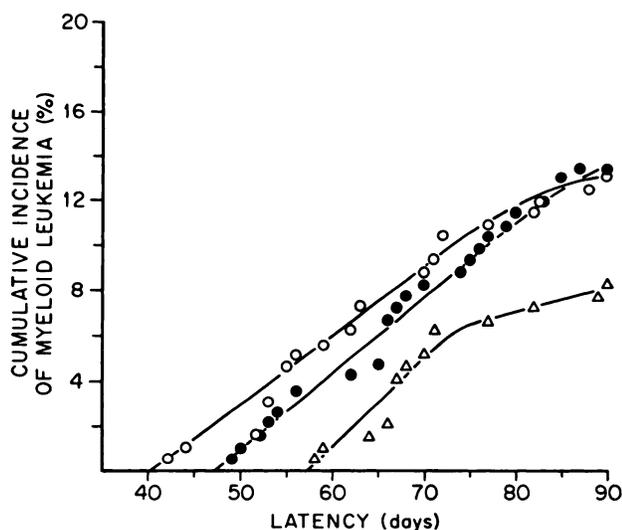


CHART 2. Cumulative incidence of myeloid leukemia in female mice, intact and gonadectomized combined, injected with leukemogenic ultracentrifugates and treated with sex hormones: ●, testosterone treated (26/192); ○, vehicle treated (25/192); and △, estrogen treated (16/192).

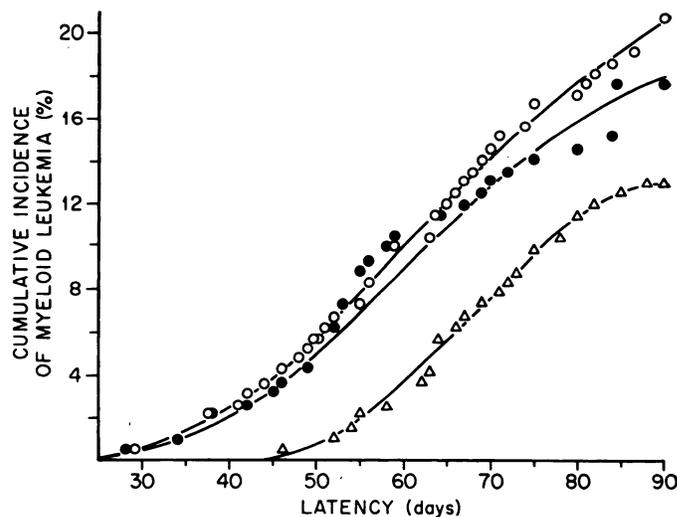


CHART 3. Cumulative incidence of myeloid leukemia in male mice, intact and gonadectomized combined, injected with leukemogenic ultracentrifugates, and treated with sex hormones: ●, testosterone treated (32/192); ○, vehicle treated (40/192); △, estrogen treated (25/192).

Testosterone did not appear to change the latency of leukemia in either sex (Table 1; Charts 2, 3).

EFFECTS OF ESTROGEN. Estrogen reduced the incidence of induced leukemia below the control level in 3 out of 4 groups (Table 1). The incidence in intact females treated with estrogen was similar to the control level (11.5% in estrogen-treatment group, 12.5% in controls), but a sizeable reduction occurred in gonadectomized females treated with estrogen (5.2% in estrogen-treatment group, 14.6% in vehicle-treated controls). Likewise, estrogen reduced the incidence in intact males (10.4% in estrogen-treated, 20.8% in controls) and gonadectomized males (15.6% in estrogen-treated, 20.8% in controls) (Table 1). Statistical comparison of combined groups indicated that the incidence in estrogen-treated mice (41/384 = 10.7%) was significantly lower

($P < 0.01$) than in vehicle-treated control mice (66/384 = 17.2%) and lower ($P < 0.05$) than in testosterone-treated mice (58/384 = 15.1%).

Estrogen consistently increased latency in females and males (Table 1; Charts 2, 3). The mean latency of leukemia in combined estrogen-treated mice (intact and castrate mice of both sexes) was significantly longer ($P < 0.02$) than that of comparable controls (69 days *vs.* 63 days) and of comparable groups treated with testosterone (69 days *vs.* 63 days).

Discussion

Although the spontaneous incidence of myeloid leukemia reaches 3-5% in RF mice, the earliest case we have noted oc-

curred in a mouse 10 months of age (8). Appearance of leukemia within 90 days after injection of ultracentrifugate into 4-week-old recipients is considered to be due to the inoculation. Evidence that the ultracentrifugates contain virus particles and are free of intact cells has been reported previously (17, 8).

The results of this study demonstrate that RF mice display a sex-dependent difference in susceptibility to induction of myeloid leukemia by ultracentrifugates. The sex-dependent difference is manifest in 2 ways: (a) by a significantly higher mean incidence of myeloid leukemia in male mice than in female mice, and (b) by a significantly shorter latent period in the males. These results are consistent with an earlier report (8) that male newborn mice given cell-free filtrates are more susceptible to induction of myeloid leukemia than females. The results are also consistent with the finding that myeloid leukemias are induced in higher frequency by x-rays in males than in females (20, 22).

Castration alone did not alter the response of either sex to induction of the disease by ultracentrifugates. It had similarly been observed that gonadectomy of mice of this strain at 28-35 days of age did not abolish the sex-dependent difference in their susceptibility to induction of leukemia by irradiation 1 week later (22). Kaplan (9) also found that gonadectomy of C57BL mice at about 30 days of age did not alter their susceptibility to induction of lymphomas by irradiation at the same age, although when they were castrated at 38-42 days of age and irradiated when 70-75 days old, the lymphoma incidence was increased in orchidectomized males (11). Since adrenals, as well as gonads, can produce estrogenic and androgenic hormones, it is conceivable that they may have maintained to some extent sex hormone production in castrated mice, at least temporarily, in this experiment and in Kaplan's.

The incidence of myeloid leukemia in testosterone-treated mice was not significantly different from that in the vehicle-treated controls. By contrast, the inhibitory effect of testosterone on lymphoma development in several strains of mice is well documented (6, 10, 16).

The estrogen-treated mice of this experiment had an incidence of leukemia lower than that of the vehicle-treated and testosterone-treated mice. These data implicate estrogen as a factor in the reduced susceptibility of female mice to myeloid leukemia. The mechanism of action of estrogen in this process remains to be elucidated. The enhancing effect of estrogen on the incidence of spontaneous lymphomas (4, 13) and on the incidence of lymphomas following irradiation (12) has been attributed to toxic effects on the thymus.

An inhibitory role of estrogen on myelopoiesis was suggested from experiments in golden hamsters (18). In the young adult hamster there is a sex difference in cellular composition of bone marrow, the male having a larger percentage of granulocytes than the female. Castration reduced the marrow granulocytes in the male and increased marrow granulocytes in the female. Testosterone treatment of intact females increased granulocyte numbers to the male level; on the other hand, estrogen treatment of the intact male decreased granulocytes. If there were a similar decrease in granulocyte numbers in RF mice treated with estrogen, fewer target cells might be present in the bone marrow for the leukemogenic agent to attack, and this might result in a reduced incidence of leukemia. Such a possibility remains to be tested.

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References

1. Barnes, W. A., and Sisman, I. E. Myeloid Leukemia and Nonmalignant Myelopoiesis in Mice. *Am. J. Cancer*, **37**: 1-35, 1939.
2. Dunn, T. B. Normal and Pathologic Anatomy of the Reticular Tissue in Laboratory Mice, with a Classification and Discussion of Neoplasms. *J. Natl. Cancer Inst.*, **14**: 1282-1433, 1954.
3. Furth, J., Upton, A. C., Christenberry, K. W., Benedict, W. H., and Moshman, J. Some Late Effects in Mice of Ionizing Radiation from an Experimental Nuclear Detonation. *Radiology*, **63**: 562-70, 1954.
4. Gardner, W. U. Influence of Estrogenic Hormones on Abnormal Growths. Occasional Publ. Am. Assoc. Advan. Sci., **4**: 67-75, 1937. (Lancaster, Pa.: Science Press.)
5. Gardner, W. U. Effect of Estradiol Benzoate and Testosterone Propionate on X-Ray Induced Leukemia in Mice. *Cancer Res.*, **10**: 219, 1950.
6. Gardner, W. U., Dougherty, T. F., and Williams, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. *Ibid.*, **4**: 73-87, 1944.
7. Gardner, W. U., and Rygaard, J. Further Studies on the Incidence of Lymphomas in Mice Exposed to X-Rays and Given Sex Hormones. *Ibid.*, **14**: 205-9, 1954.
8. Jenkins, V. K., and Upton, A. C. Cell-free Transmission of Radiogenic Myeloid Leukemia in the Mouse. *Ibid.*, **23**: 1748-55, 1963.
9. Kaplan, H. S. Influence of Thymectomy, Splenectomy, and Gonadectomy on Incidence of Radiation-induced Lymphoid Tumors in Strain C57 Black Mice. *J. Natl. Cancer Inst.*, **11**: 83-90, 1953.
10. Kaplan, H. S., and Brown, M. B. Inhibition by Testosterone of Radiation-induced Tumor Development in Intact and Castrate Adult Mice. *Cancer Res.*, **11**: 706-8, 1951.
11. Kaplan, H. S., Nagareda, C. S., and Brown, M. B. Endocrine Factors and Radiation-induced Lymphoid Tumors in Mice. *Recent Prog. Hormone Res.*, **10**: 293, 1954.
12. Kirschbaum, A., Shapiro, J. R., and Mixer, H. W. Synergistic Action of Leukemogenic Agents. *Cancer Res.*, **13**: 262-68, 1953.
13. Lacassagne, A. Sarcomes lymphoides apparus chez des souris longuement traitée par des hormones oestrogènes. *Compt. Rend. Soc. Biol.*, **126**: 193-95, 1937.
14. Law, L. W. Effects of Gonadectomy and Adrenalectomy on Appearance and Incidence of Spontaneous Lymphoid Leukemia in C58 Mice. *J. Natl. Cancer Inst.*, **8**: 157-59, 1947.
15. McEndy, D. P., Boon, M. C., and Furth, J. On the Role of Thymus, Spleen and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice. *Cancer Res.*, **4**: 377-83, 1944.
16. Murphy, J. B. Effects of Castration, Theelin and Testosterone in a Rockefeller Institute Strain of Mice. *Ibid.*, **4**: 622-24, 1944.
17. Parsons, D. F., Upton, A. C., Bender, M. A., Jenkins, V. K., Nelson, E. S., and Johnson, R. R. Electron Microscope Observations on Primary and Serially Passaged Radiation-induced Myeloid Leukemias of the RF Mouse. *Ibid.*, **22**: 728-36, 1962.

18. Ravines, H. T. Effect of Castration, Hypophysectomy, Androgens and Estrogens on the Spleen and Bone Marrow of Golden Hamsters. *Lab. Invest.*, 10: 341-53, 1961.
19. Upton, A. C. Studies on the Mechanism of Leukaemogenesis by Ionizing Radiation. *Ciba Found. Symp. Carcinogenesis: Mechanisms Action*, p. 249, 1959.
20. Upton, A. C., and Furth, J. The Effects of Cortisone on the Development of Spontaneous Leukemia in Mice and on Its Induction by Irradiation. *Blood*, 9: 686-95, 1954.
21. ———. Host Factors in the Pathogenesis of Leukemia in Animals and in Man. *Proc. Natl. Cancer Conf.*, 3rd, 1956 (1957), pp. 312-24.
22. Upton, A. C., Wolff, F. F., Furth, J., and Kimball, A. W. A Comparison of the Induction of Myeloid and Lymphoid Leukemias in X-radiated RF Mice. *Cancer Res.* 18: 842-48, 1958.