Abrogated Thymoma Development and Increased Amyloid Development in Estrogenized Mice Grafted Spleen and Bone Marrow Cells[1] (36004)

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(Introduced by N. A. Thorn)

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Lifelong treatment of BALB/c mice with estradiol benzoate leads to a general lymphocyte depletion (1), thymic cyst formation (2, 3), and often thymic lymphoma (4–6).

Working on the assumption that an immunodepression is essential to the oncogenic effect of estrogen, we have tried to influence the effects of estrogen by repeated grafting of syngeneic cells and tested cellular immunity of estrogen-treated mice.

Materials and Methods. Inbred BALB/c and AKA mice were used. Estradiol benzoate in aquous suspension (Ovex, Løvens Kemiske Fabrikker, Ballerup, Denmark) was administered once a month from 2 months of age and throughout life by injecting 0.25 mg subcutaneously (sc).

Single cell suspensions of the cells obtainable from the spleen and bone marrow of both femurs from 2-month-old syngeneic untreated donor mice were made up in phosphate buffered saline by squeezing through a mesh. Trypan blue stain assured the viability of more than 90% of the cells. All cells obtained from one donor (about 8×10^7) were given intraperitoneally to one recipient 14 days after each inoculation of estrogen.

Estrogen-treated mice were sacrificed when ill. Control animals were given saline sc. Lung, liver, spleen, kidney, peripheral lymph nodes, thymus, and thyroid gland were taken for microscopy and stained with hematoxylin–eosin and periodic acid–Schiff (PAS). Blood was taken for leukocyte counting.

To measure the blast forming capacity of lymphocytes from the peripheral blood (1) cultures containing 2×10^6 lymphocytes were given 0.025 ml of PHA (Burroughs, Welcome & Co., London, Batch K 7915). After 3 days 2.5 nCi of tritium-labeled thymidine was added and the incorporation determined 6 hr later. The conditions of culture have been described elsewhere (7). The blood was drawn from male mice pretreated with estrogen for 6 months and from saline-treated controls, estrogen was last given 1 month prior to the test.

Skin grafts (2×3 cm) were obtained from the backs of 2-month-old C3H mice, deprived of subcutaneous fat and fixed by six stitches to the backs of BALB/c mice pretreated with estrogen or saline for 6 months. The last inoculation of estrogen occurred 1 month before the grafting. Recipients which on autopsy showed signs of leukemia were not included in the material.

Results. All 47 estrogen-treated mice showed depletion of lymphocytes in thymus, spleen and lymph nodes. Peripheral leukocyte counts at time of death were slightly depressed (from about 4000/mm^3 in controls to about 3000/mm^3 in estrogen-treated mice). The percentage of granulocytes was about 40% in both estrogen-treated and saline-treated mice. All male mice had large interstitial cell testicular tumors following treatment (2).

Estrogen treatment in both sexes lead to appearance of thymus cysts. In addition most males and some female mice developed large thymic lymphomas (Table I) (Fig. 1), 10^4–10^5 leukocytes/mm^3 peripheral blood, and leukemic lymphocyte infiltrations in

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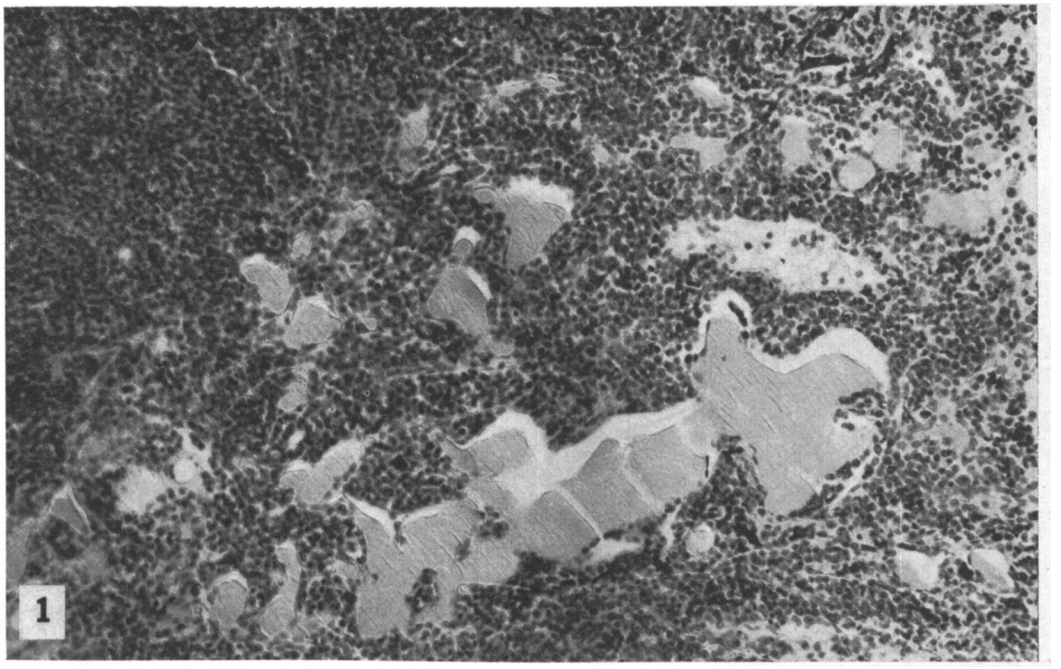

FIG. 1. Thymus from 11-month-old BALB/c mouse given estrogen for 8 months. Numerous medullary cysts are seen together with massive infiltration of malignant lymphocytic cells. PAS technique, ×525.

most organs (3). The mean survival time of both males and females was significantly ($p<.01$) shorter than in untreated mice (2). Cases of diarrhea, as seen with higher dose of estrogen (3), were not observed. Bone marrow cavities were reduced in size due to thickening of the bone.

Grafting of mice with syngeneic spleen marrow cells during the estrogen treatment significantly reduced the incidence of thymic leukemias (Table I), best demonstrated in the males where the mean survival time was unaffected by the inoculation of cells. Thymus cysts were present in both leukemic (Fig. 1) and nonleukemic animals, as were testicular tumors. The amount of cysts was not detectably altered by grafting with cells and the thymus cellularity also seemed unchanged by grafting of cells. Spleen amyloid staining positively with alkaline Congo red was confined to animals free of thymoma. Amyloidosis occurred most frequently in the group: estrogen + cells with the lowest incidence of thymoma.

A preliminary investigation of lymphocytes from untreated mice established the optimum concentration of PHA for inducing blast formation (Table IIa). Using that concentration of PHA lymphocytes from 9 estrogen-treated and 10 saline-treated mice were tested. Very similar reactions were found in the two groups (Table IIb).

The C3H skin grafts were rejected after 28 ± 4 days when the BALB/c recipient had been pretreated with estrogen; whereas 17 saline-treated BALB/c recipients rejected the grafts after 22 ± 3 days. No rejection was found when the mice were grafted with BALB/c skin.

An attempt to accelerate thymoma development in AKA mice by administering estrogen according to our schedule was successful as far as treated mice had a reduced mean survival time and showed thymus cysts, but still showed an unchanged high incidence of thymic lymphomas (Table III).

Considering the possibility that spontaneous thymomas in AKA mice may be elicited by some mechanism similar to that operating in estrogen-treated mice, we grafted un-

TABLE I. Influence of Grafting with Syngeneic Spleen and Bone Marrow Cells from 2-Month-Old Untreated Donors on Leukemia Development in Estrogen-Treated BALB/c Male and Female Mice.

Estrogen-treated mice killed when ill; saline-treated controls killed 12 months old (not ill).

Treatment	Sex	Number				Mean survival time (months)
		Total		No.	(%)	
Estrogen	Male	30	Thymoma	12	40	11
			Spleen amyloidosis	4	8	9
			Thymoma + amyloidosis	0	0	
			Other animals	14	52	10
	Female	30	Thymoma	6	20	10
			Spleen amyloidosis	1	3	10
			Thymoma + amyloidosis	0	0	
			Other animals	23	77	11
Estrogen + cells	Male	10	Thymoma	1	10	11
			Spleen amyloidosis	3	30	9
			Thymoma + amyloidosis	0	0	
			Other animals	6	60	10
	Female	10	Thymoma	0	0	
			Spleen amyloidosis	3	30	9
			Thymoma + amyloidosis	0	0	
			Other animals	7	70	7
Saline + cells	Male	10	Thymoma	0	0	
			Spleen amyloidosis	1	10	12
			Thymoma + amyloidosis	0	0	
			Other animals	9	90	12
	Female	10	Thymoma	0	0	
			Spleen amyloidosis	0	0	
			Thymoma + amyloidosis	0	0	
			Other animals	10	100	12

treated AKA mice with syngeneic cells (Table IV). This treatment did not interfere with "spontaneous" thymoma development or survival time in AKA mice. It is noteworthy that spleen amyloidosis occurred in one fourth of the males and that these males were all free of thymoma or other leukemias.

Discussion. The high incidence of thymic lymphomas in mice given protracted course of estrogen is well known (4–6). Our observations show that with the schedule used by us on BALB/c mice a sex difference in response occurs. This may relate to the known immunologic superiority of female mice (8, 9) and production of hormones in estrogen-induced testicular tumors (10). Male Swiss mice have also been found more susceptible to skin tumorgenesis by 7,12-dimethylbenz[*a*]anthrazene than female mice (11).

Protection against the occurrence of estrogen-induced thymic lymphoma by inoculation of syngeneic spleen and marrow cells has not been reported previously. Our observation brings hormone-induced thymoma in line with thymic lymphomas induced by X-irradiation (12) and chemical carcinogen (13) both of which can be prevented by grafting with syngeneic spleen and bone marrow cells (12, 13).

Estrogen-treated mice clearly have a reduced number of lymphoid cells throughout the body (2). Blood lymphocytes are, however, only moderately reduced in number and their response to PAH does not point to an inferior function of the lymphocytes in circulation. A more important alteration may be a reduced supply of marrow-derived cells to the thymus (7, 14) due to the mitosis-inhibiting

effect of estrogen (15, 16) and/or the reduced volume of the marrow cavity in estrogen-treated mice (17). The latter possibility is rendered less likely by the observation that there is no correlation between the tendency to skeletal changes and thymoma development in different mouse strains after estrogen treatment (18). The slightly prolonged rejection time after grafting of allogeneic skin to estrogen-treated animals indicates a reduced immune responsiveness of the estrogenized animal (19). This could be of importance for the development of lymphoma since antigen-treated BALB/c mice have a greatly enhanced leukemia incidence only when immune-suppressed (20).

TABLE II. PHA Induced Blast Formation in Peripheral Lymphocytes from Estrogen-Treated and Saline-Treated BALB/c Mice.

a. Effect of Various Doses of PHA on Incorporation of Thymidine in 2.5 × 10^6 Peripheral Lymphocytes from Normal BALB/c Mice.

0.025 ml PHA dilution	10 cpm (each dilution tested with 4 cultures)
1:3	8–17
1:6	21–27
1:12	31–67
1:18	42–69
1:24	52–68
1:36	36–48
1:48	19–24

b. A Comparison of the Rate of Thymidine Incorporation in Lymphocytes from Mice Pretreated for 6 Months with Estrogen or Saline when Stimulated with PHA (1:24).

	Estradiol treated		Saline treated	
	Culture 2 × 10^6 cells	10^3 cpm	Culture 2 × 10^6 cells	10^3 cpm
Blood	1	20	1	19
from	2	14	2	15
6 mice	3	17	3	19
pooled	4	18	4	19
in each	5	18	5	18
culture	6	20	6	18
	1.5 × 10^6 cells		1.5 × 10^6 cells	
Blood	1	8	1	7
from	3	9	2	13
individ-	3	5	3	8
ual mice			4	11

The pathogenesis for estrogen-induced testicular tumors may differ from that of estrogen-induced thymomas as testicular tumors appeared regardless of grafting with syngeneic cells or not.

That the estrogen-induced thymus cysts are not important for thymoma development is derived from the following observations:

1. Untreated BALB/c mice of more than 2 years of age may show thymus cysts, but thymomas are extremely rare in untreated old BALB/c mice (2).

2. Abrogation of the thymoma-inducing effect of estrogen by grafting with syngeneic cells did not prevent cyst development.

In addition to a reduction in thymoma incidence syngeneic cells also caused an increase in the incidence of spleen amyloidosis in estrogenized mice. This amyloidosis, which only occurred in mice free of thymoma, must be attributed to a combined effect of cells and estrogen as amyloid was found infrequently when cells were given with saline only. A great part of the grafted cells are probably quickly being removed by host macrophages. Perhaps estrogen may increase the breakdown of donor cells (3) and, in addition, impede the division of recipient macrophages (15, 16), something which may be relevant to amyloid development (22, 23).

Whether thymoma prevention and amyloid promotion is causally related or independent reflections of alterations following the grafting of cells is undecided.

The possibility that spontaneous lymphoma development in AKA mice in analogy with estrogen-induced thymomas in BALB/c mice may be precipitated by a lack of cells present in spleen and marrow of young mice was not borne out in our experiment. Law (21) has pointed to the state of the thymic reticular tissue as decisive for spontaneous thymoma development in AKA mice.

Grouped male mice of AKA strain often bite each other and amyloid development is usually considered a result of such antigenic stimulation (24). One likely factor for the total prevention of thymoma development in

TABLE III. Thymic Lymphoma in Estrogen-Treated and Saline-Treated AKA Mice. The mice in all groups were sacrificed when clinically ill.

Treatment	Sex	Thymoma/total		Survival time (months)	
		No.	(%)	Thymoma	Total
Estrogen	Male	8/13	60	7	6
	Female	6/10	60	5	6
Saline	Male	4/10	40	10	11
	Female	12/16	75	8	9

amyloid AKA mice therefore might be chronic antigenic stimulation of thymus (25).

As estrogen accelerated the appearance of AKA thymomas, which probably are viral induced and since virus has been demonstrated in thymomas of estrogen-treated RF mice (26), estrogen may well cause thymic lymphomas in BALB/c mice by facilitating growth of a leukemia virus.

This possibility is currently under investigation.

Summary. Adult BALB/c mice treated monthly subcutaneously with 0.25 mg estradiol benzoate, had a high incidence of thymic lymphomas and more so in males than in females.

Administering of syngeneic spleen and bone marrow cells to estrogenized mice (i) protected against development of thymic lymphomas; (ii) increased the incidence of spleen amyloidosis; and (iii) was without influence on development of thymic cysts and testicular tumors.

Blood lymphocytes from estrogenized mice reacted normally to phytohemagglutinin, but the mice had a prolonged rejection time of allogeneic skin graft.

Spontaneous thymoma development in AKA mice was not influenced by grafting syngeneic cells.

Thymoma and spleen amyloidosis occurred as mutually exclusive qualities in both BALB/c and AKA mice.

TABLE IV. Thymic Lymphoma and Spleen Amyloidosis in AKA Mice Grafted ip Monthly with Syngeneic Spleen and Marrow Cells from 2-Month-Old Syngeneic Donors.

Treatment	Sex	Number				Mean survival time (months)
		Total		No.	(%)	
Cells	Male	22	Thymoma	12	54	9
			Spleen amyloidosis	5	23	9
			Thymoma + amyloidosis	0	0	
			Other	5	23	9
	Female	24	Thymoma	24	100	9
			Spleen amyloidosis	0	0	
			Thymoma + amyloidosis	0	0	
			Other			
Saline	Male	42	Thymoma	26	62	9
			Spleen amyloidosis	11	26	8
			Thymoma + amyloidosis	0	0	
			Other	5	12	6
	Female	44	Thymoma	43	98	8
			Spleen amyloidosis	1	2	6
			Thymoma + amyloidosis	0	0	
			Other	0	0	

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