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MECHANISMS OF ESTROGEN-INDUCED MYELOTOXICITY: EVIDENCE OF THYMIC REGULATION

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Abstract — Mice exposed to pharmacological levels of steroidal and nonsteroidal estrogens including α-dienestrol, 17β-estradiol, and diethylstilbestrol demonstrate bone marrow hypocellularity, and decreased numbers of pluipotent hemopoietic stem cells. Hormones with little estrogenic activity including testosterone and progesterone failed to induce myelotoxicity as did nonestrogenic metabolites of DES. Myelotoxicity associated with estrogen exposure is regulated by a complex bimodal mechanism. One of these mechanisms is mediated through the thymus since surgical thymectomy abolished the ability of estrogens to suppress CFU proliferation. Furthermore, supernatants of thymic epithelial cells cultured in the presence of estradiol were capable of inhibiting CFU-GM colony formation. Specific myelotoxic events can also be disassociated chemically by testing weakly estrogenic compounds such as zearalanol which shows different sensitivity on cytoxic and antiproliferative events. Myelotoxicity is not mediated indirectly through the ovary or adrenal gland. That the initial events in estrogen-induced myelotoxicity may be mediated through a receptor mechanism was suggested by the ability of antiestrogens to induce antagonism when administered prior to estradiol and the presence of estrogen binding components in lymphoreticular tissues including the thymus and bone marrow. These studies suggest that reduced CFU kinetics observed following estrogen exposure is, in part, due to alterations in regulatory factors produced by thymic epithelial cells in response to a specific estrogen stimulus. Estrogens may also influence bone marrow functions through non-thymic mechanisms at higher dose levels.

Blood cells arise from proliferation of multitiered stem cells. Hematopoiesis occurs primarily within the bone marrow, although in rodents the spleen may serve as a major secondary hematopoietic organ. This process is regulated by both positive and negative factors including colony stimulating factor (CSF), chalones, prostaglandins, thymic hormones, etc (rev. by Cline & Gold, 1979). Since hematopoiesis represents a highly organized and regulated cellular network which undergoes continuous proliferation and differentiation, it can serve as a sensitive organ-system for determining chemically-induced cellular injury (Boorman, Luster, Dean & Campbell, 1982). Furthermore, procedures are readily available for morphological and functional characterization of both the multipotential stem cell or colony forming unit- spleen (CFU-S) as well as committed progenitor cell populations (Cline & Gold, 1979).

Adult mice exposed to exogenous estrogens such as estradiol or diethylstilbestrol (DES) exhibit thymic atrophy, depressed cell mediated immunity, induction of inflammatory macrophages and depressed natural killer cell activity (e.g. Ahlquist, 1976; Kalland, 1982; Kalland, 1980; Luster, Boorman, Dean, Lawson, Wilson & Haseman, 1981; Seaman, Merigant & Talal, 1979). In addition, DES or estradiol exposure can adversely affect hematopoiesis (Fried, Tichlar, Dennenberg, Barone & Wang, 1974; Boorman, Luster, Dean & Wilson, 1980; Reisher, 1966). Estrogen induced myelotoxicity is characterized by bone marrow hypocellularity and depressed numbers of CFUs (Fried *et al.*, 1974; Boorman *et al.*, 1980). Decreased bone marrow erythropoiesis also occurs although compensatory

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increased splenic erythropoiesis is observed several days following cessation of estrogen administration. Splenic compensation does not occur for CFU-S or CFU-GM progenitor cells which remains depressed in both the bone marrow and spleen for several weeks (Fried *et al.*, 1974; Boorman *et al.* 1980). Myelotoxicity occurs in the absence of estradiol-induced osteoproliferation indicating that cell kinetics are affected by estrogen and not simply decreased marrow volume (Seaman, Gindhart, Greenspan, Blackman & Talal, 1979).

The present studies demonstrated that marrow hypocellularity and depressed CFU proliferation induced by administration of pharmacological levels of estrogens are controlled by separate mechanisms, the latter being directly influenced by thymic factors, although both events can be directly related to uterotrophic responses (estrogenic activity). Preliminary data also suggested that many of the observed estrogen effects may depend upon the relative binding interaction of the estrogenic compounds to various target tissues or cells which could then mediate hormonal effects on selective lymphoreticular cells. The degree of thymic atrophy was included as an *in vivo* marker in many of these studies since this appears to be one of the most sensitive non-uterotrophic responses for estrogen exposure in mice (Boorman *et al.*, 1980; Kalland, Fossberg & Fossberg, 1978; Sobhon & Jirasttham, 1974).

EXPERIMENTAL PROCEDURES

Mice

Female B6C3F1 (C57B16/J × C_3H/HeJ) mice, 6–8 weeks of age and weighing 18–21 g were obtained from the NCI production contracts (Charles River, Portage, MI). Animals were maintained on a 12 h light/dark cycle at 68 to 73°F and provided sterile food (Zeigler Bros., Inc., Gardner, PA) and water *ad libitum*. All surgical procedures including adult thymectomy (ATX), adrenalectomy (ADRX), and ovariectomy (OVX) were performed under pentabarbital anesthesia.

Chemicals

Diethylstilbestrol (DES, Lot 29C-0318); 17β-estradiol (Lot 199C-0324); testosterone (Lot 81F-0308) and α-dienestrol (E,E-DIES, Lot 37C-0369) were from Sigma Chemical Co., St. Louis, MO. Zearalanol (P-1496; Lot 20-179) was a gift from Dr. M. Bachman of International Minerals and Chemical Corporation (Terre Haute, IN). Nafoxidine-HCl was a gift from Dr. P. W. O'Connell of the Upjohn Company (Kalamazoo, MI). Tamoxiphen was a gift from Dr. D. H. McCurdy, Stuart Pharmaceuticals (Wilmington, DL). Dr. M. Metzler (University of Wurzburg, West Germany) kindly provided the β-dienestrol (Z,Z-DIES).

Exposure regimens

Mice were exposed to test compounds daily for 5 consecutive days. The materials were initially dissolved in corn oil and diluted such that each mouse received a volume of 0.1 ml subcutaneously in the dorsal cervical region. Controls received an equal volume of corn oil. In studies using estrogen antagonists, mice routinely received 8.0 μmoles of the antiestrogen over a 3 day period and 6.0 μmoles of 17β-estradiol administered simultaneously during the last two days of this exposure regimen. Mice were examined 4 days following cessation of treatment unless otherwise stated.

Bone marrow cellularity

Marrow cells from mice were aseptically collected from both femurs by dissecting the femor free of attached tissue, cutting both ends at the epiphysis and flushing the shaft with 2 ml of cold Hank's balanced salt solution (HBSS) containing 10 units of heparin per ml (preservative free) using a 3 ml syringe and a 23 gauge needle. Dispersions were made by passing the cells through 23 and 25 gauge needles successively. The number of nucleated cells was determined with a Coulter Counter. Cell viability as assessed by trypan blue exclusion was always >95%.

Colony forming units in culture (CFU-GM)

Bone marrow macrophage-granulocyte progenitor cells were assayed using a modification of a previous method (Bradley & Metcalf, 1966). Briefly 1×10^5 nucleated femoral marrow cells in RPMI 1640 culture media were plated in 35 × 10 mm Falcon tissue culture dishes (BioQuest, Cockeysville, MD). The media was supplemented with 1.5% methylcellulose (Dow Chemical Co., Midland, MI), fresh frozen 10% human AB serum (Irvine Scientific, Irvine, CA), 2 mM L-glutamine, 0.5 μg/ml gantamycin and 10% mouse lung conditioned media (MLCM). MLCM was prepared as previously described (Sheridan, Metcalf & Stanley, 1975) with some modifications. In order to prepare MLCM, mice received 10 μg endotoxin (Lipopolysaccharide *E. coli*, Sigma) by intravenous injection, sacrificed 4 h later by cervical dislocation, and the lungs aseptically removed. Lung fragments were cultured for 48 h in serum free media and supernatants collected. The conditioned

media was filtered through 0.2 micron filters (Sybron/Nalge Corp., Rochester, NY) that had been pretreated with polyethylene glycol to prevent loss of colony stimulating activity. The concentration needed for optimal stimulation of macrophage-granulocyte progenitor cells was determined and the conditioned media stored at 4°C until used.

Culture plates were incubated at 37°C, 7.5% CO_2 in air in a humidified atmosphere for 7 days. The plates were stained with methylene blue (Loefflers, Modified, Fisher). Total colonies (40 or more cells) per plate were counted using a stereomicroscope. Four plates were counted for each bone marrow determination.

Colony forming units in spleen (CFU-S)

The assay for bone marrow stem cells using the spleen colony method was originally described by Till & McCulloch (1961). The recipient mice were B6C3F1 males, 6 – 10 weeks of age, that had received 800 rad of total body irradiation at 40 rad/min (^{137}Cesium Irradiator, Model 431). Each recipient received 5×10^4 nucleated bone marrow cells i.v. within 24 h of irradiation. Six to ten recipients were used for each donor. Eight days later, the spleens were removed, placed in Bouin's fixative and spleen colonies counted under a stereomicroscope. Histological sections were made of various colonies to confirm their myeloid nature.

^{59}Fe uptake assay

The ^{59}Fe uptake assay is a modification of the method described by Boggs, Boggs, Cherevenick & Patrene (1980). Control and treated mice (4/group) were injected intraperitoneally with 0.5 μCi of ^{59}Fe (specific activity 12.2 mCi mg^{-1}, New England Nuclear, Boston, MA) in 0.5 ml of 0.85% NaCl solution. Eighteen hours later, the mice were sacrificed and radioactivity in the spleen and leg from each mouse was determined in a Packard Autogamma Counter (PRIAS Model PGD).

Estrogen receptor assay

Tissues were removed from intact or ovariectomized mice, placed in cold TE-P buffer (10 mM Tris, 1.5 mM disodium EDTA and 0.1 mM PMSF) and homogenized at a concentration of 75 mg wet weight/ml buffer. Bone marrow and thymocyte cell suspensions were prepared in Hank's balanced salt solution (HBSS), centrifuged, repeatedly washed in TE-P buffer and homogenized with 20 strokes in a dounce ounce homogenizer (pestle A) at a concentration of 1.5×10^7 cells/ml TE-P buffer. Homogenates were centrifuged at 1000 × g for 15 min, the supernatant decanted and recentrifuged at 105,000 × g for 50 min. Aliquots of the resulting supernatant (cytosol) were incubated with 5 nM ^{3}H-estradiol and specific binding activity determined as previously described (Korach, 1979). Protein samples of the cytosol preparations were measured in duplicate by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951).

Thymic epithelial cell cultures

Thymic epithelial cell cultures from C57Bl mice were kindly provided by Dr. W. Greenlee (Chemical Industry Institute of Toxicology, Research Triangle Park, NC) and were prepared as previously described (Boniver, DeCleve, O'Dailey, Honsik, Lieberman & Kaplan, 1981). Primary cultures were maintained in the presence of Mycostatin and MEM-d-valine to inhibit the growth of macrophages and fibroblasts, respectively. Various concentrations of 17β-estradiol or vehicle (0.1% EtOH) were incubated for 48 h at 37°C with thymic epithelial cell monolayers. The supernatants were collected and added to CFU-GM colony assays (10% v/v) at the initiation of the culture period. To determine the direct effect of estradiol on CFU-GM proliferation, equimolar concentrations of 17β-estradiol were added directly to the CFU-GM culture.

Statistical analysis

The Mann – Whitney U test and Student's *t*-test were employed to assess the significance of treatment effects while dose – response trends were determined by Jonckheere's test.

RESULTS

Exposure to DES or 17β-estradiol caused a dose related decrease in thymic weights, bone marow cellularity, CFU-S numbers and CFU-GM numbers in B6C3F1 mice (Table 1). In some instances exposure to DES was more myelotoxic than estradiol at equimolar exposure levels. Depressed marrow erythropoiesis, as determined by ^{59}Fe-uptake, was not observed with estradiol, although erythropoiesis was compromised following exposure to DES. There was no histological evidence of osteoproliferative related treatment effects. At higher dosage levels of DES there was an apparent compensatory increase in splenic erythropoiesis. This was also supported histologically by marked hematopoiesis in the spleen. Significantly decreased thymic weights occurred in all treatment groups except the low dose estradiol

Table 1. Thymic atrophy and bone marrow functions in estradiol and DES treated mice

Treatment	Total dosage* (μmoles)	Thymic atrophy (% of Controls)	Bone marrow cellularity/Femur ($\times 10^{-6}$)	CFU-GM/ 10^5 Cells	CFU-S/ 5×10^4 Cells	^{59}Fe-Uptake (CPM) Femur	Spleen
Vehicle	—	—	20.0 ± 1.1†	75 ± 1	17.5 ± 0.3	682 ± 120	351 ± 82
Estradiol	0.07	7↓	14.3 ± 1.2‡	69 ± 1‡	16.0 ± 0.4‡	716 ± 46	397 ± 41
	0.7	18↓‡	13.0 ± 0.6§	60 ± 1§	14.8 ± 0.4§	618 ± 126	260 ± 27
	2.8	40↓§	13.6 ± 1.0§	54 ± 3§	13.3 ± 0.4§	514 ± 61	290 ± 55
	5.6	39↓§	12.2 ± 0.9§	51 ± 2§	11.8 ± 0.3§	563 ± 60	425 ± 70
DES	0.07	39↓§	14.6 ± 1.2‡	63 ± 1§	16.5 ± 0.3	708 ± 87	307 ± 29
	0.7	47↓§	13.3 ± 1.2§	59 ± 1§	15.1 ± 0.3§	424 ± 15	518 ± 205
	2.8	66↓§	12.2 ± 0.8§	59 ± 1§	12.8 ± 0.1§	365 ± 19‡	759 ± 164‡
Dose Response (P)		<0.01	<0.01	<0.01	<0.01	<0.01	NS

*Mice were exposed daily over a 5 day period and tested 4 days following the last exposure.
†Each value represents mean ± SEM of 5 mice/group.
‡Significantly different from controls at $P<0.05$.
§Significantly different from controls at $P<0.01$.

group. The thymus was histologically characterized in DES and estradiol treated mice by a progressive reduction in thymic cortical lymphocytes without prominent pkynosis or karyorrhexis. These results confirm and extend those which have been previously reported (3,8,9).

The results in Table 2 summarize the myelotoxicity and thymic atrophy that occurred in mice exposed to equimolar (2.8 μmoles) concentrations of various steroidal and nonsteroidal hormones or their metabolites. The most pronounced effects on thymic atrophy and bone marrow functions occurred following exposure to the most estrogenic compounds; mainly estradiol, DES and EE-DIES which caused greater than 50% reduction in thymic weights and 30% decrease in bone marrow function. Zearalanol, which is considerably less estrogenic than estradiol in stimulating uterine growth, was equally effective in suppressing CFU-GM and CFU-S numbers but less effective in causing thymic atrophy and marrow hypocellularity. Testosterone, ZZ-DIES and progesterone which have no appreciable estrogenic activity had no demonstrable effect on the thymus or bone marrow.

In order to further examine the relationship between estrogenicity and myelotoxicity, several antiestrogens were evaluated for their antagonistic effects on estrogen myelotoxicity. As can be seen in Table 3, both antiestrogens, Tamoxiphen and Nafoxidine when administered prior to estradiol,

Table 2. Thymic atrophy and myelotoxicity following exposure to steroidal and nonsteroidal compounds with various estrogenic activity

Treatment*	Thymic weight[b] (% of Controls)	Bone marrow cellularity/femur ($\times 10^{-6}$)	CFU-GM/ 10^5 cells	CFU-S/ 5×10^4 cells	Relative‡ *in vivo* uterotrophic activity	Relative‡ *in vitro* estrogen binding activity
Vehicle	—	20.5 ± 0.9	73 ± 1	16.9 ± 0.3	—	—
Estradiol	55↓†	14.6 ± 0.9§	67 ± 2§	13.5 ± 0.3§	1	1
DES	79↓§	14.3 ± 1.1§	54 ± 2§	10.2 ± 0.4§	1	0.7
E,E-DIES	79↓§	13.1 ± 0.5§	63 ± 1§	11.6 ± 0.2§	0.07	5.0
Z,Z-DIES	8↓	19.0 ± 1.4	72 ± 2	15.8 ± 0.3	<0.001	360
Zearalanol	25↓§	17.7 ± 0.9	67 ± 2§	11.6 ± 0.3§	0.01	500
Testosterone	14↓	17.2 ± 1.3	70 ± 2	15.9 ± 0.2	<0.001	>1000
Progesterone	4↓	17.2 ± 1.4	71 ± 3	16.1 ± 0.2	<0.001	>1000

*Adult female mice were exposed to a total of 2.8 μmoles of the test compound over a 5 day period and tested 4 days following the last treatment.
†Each value represents the mean ± SEM of at least 6 mice/group.
‡Relative to *in vitro* activity of 17β-estradiol (From reference Duax and Weeks, 1980; Korach *et al.*, 1978; Schmidt *et al.*, 1976).
§Significantly different from controls at $P<0.01$.

Table 3: Inhibition of estrogen-induced myelotoxicity by antiestrogens

Estrogen treatment*	Antiestrogen treatment*	Thymic atrophy (% Change)	Bone marrow cellularity/ Femur ($\times 10^{-6}$)	CFU-S/ 5×10^4 Cells
Vehicle	Vehicle	—	20.9 ± 0.9	17.4 ± 0.2
Vehicle	Tamoxiphen	40↓‡	18.7 ± 0.6	17.0 ± 0.2
Estradiol	Tamoxiphen	54↓‡	19.1 ± 0.9	17.1 ± 0.2
Vehicle	Nafoxidine	25↓†	19.7 ± 0.8	17.0 ± 0.2
Estradiol	Nafoxidine	39↓‡	18.5 ± 0.8	17.0 ± 0.2
Estradiol	Vehicle	68↓‡	15.7 ± 0.9‡	14.2 ± 0.4†

*Mice were treated with a total of 8.0 μmoles of antiestrogen over a 3-day period. On the last two treatment days, mice were simultaneously administered a total of 6 μmoles of 17β-estradiol and tested 4 days later. Each value represents the mean ± SEM of 6 mice/group except controls (vehicle – vehicle) which represents 12 mice.
†Significantly different from controls at $P < 0.05$.
‡Significantly different from controls at $P < 0.01$.

Table 4. The effect of adrenalectomy (adrx) or ovariectomy (ovx) on estrogen induced thymic atrophy and myelotoxicity*

Treatment*	Surgical procedure	Thymic atrophy (% Change)	Bone marrow cellularity/ femur ($\times 10^{-6}$)	CFU-S/ 5×10^4 cells
Corn Oil	None	—	21.8 ± 0.7	17.5 ± 0.3
	Sham-adrx	5↓	20.0 ± 0.6	17.1 ± 0.1
	Adrx	48↑†	21.7 ± 1.1	17.1 ± 0.1
	Sham-ovx	12↑	21.6 ± 1.0	17.4 ± 0.1
	Ovx	34↑†	20.4 ± 0.7	17.6 ± 0.1
Estradiol	None	75↓†	12.9 ± 0.3†	14.3 ± 0.4†
	Sham-adrx	61↓†	13.9 ± 0.4†	13.4 ± 0.1†
	Adrx	32↓†	14.3 ± 0.7†	13.5 ± 0.2†
	Sham-ovx	71↓†	10.5 ± 0.3†	13.9 ± 0.3†
	Ovx	50↓†	11.9 ± 0.6†	15.0 ± 0.4†

*Adult female mice were adrenalectomized or ovariectomized and subsequently maintained on drinking water containing high salt (0.8%) and glucose (1.0%). Five days following adrenalectomy or ovariectomy animals were exposed to either corn oil or 2.8 μmoles of 17β-estradiol over a 5 day period and tested 4 days later.
†Significantly different from controls at $P < 0.05$.

caused almost complete inhibition of estrogen-induced myelotoxicity. Marrow cellularity and CFU numbers were similar to control values in mice administered the antiestrogens alone. In contrast, when thymic atrophy was examined, the antiestrogens appeared to act as only partial antagonist, and if administered alone, caused some atrophy. However, although thymic atrophy is a sensitive qualitative indicator for estrogen exposure, weight variation is not a sensitive quantitative indicator.

During routine histological examination, pathological alterations in the adrenals were noted in DES, estradiol and E,E-DIES treated mice and was characterized by a moderate degree of cytoplasmic vacuolization in the zona fasciculata. Thus, a series of experiments involving adrenalectomized mice were performed to determine if the myelotoxicity was indirectly related to altered adrenal function (e.g. increased steroid secretion). Adrenalectomy or sham adrenalectomy failed to influence estrogen induced myelotoxicity with all parameters in estrogen exposed mice comparably affected regardless of surgical manipulations (Table 4). Furthermore, bone marrow functions were not influenced by ovariectomy since both intact and ovariectomized mice were equally affected following estradiol treatment. Both adrenalectomy and ovariectomy, without exogenous estrogen, caused thymic hyperplasia. However, estrogen administration was equally effective in inducing thymus atrophy in surgically altered as well as intact mice.

In order to further examine the role of estrogen receptors in myelotoxicity, specific estrogen binding capacities of cytosol preparations from various

Table 5. Cytoplasmic estrogen binding in various tissue

Sample	Specific binding (fmoles/mg cytosol protein)*
Thymus	42 ± 14*
Bone marrow	59 ± 24
Uterus	387 ± 24
Esophagus	<2.0

*Values represent mean ± S.D. from triplicate samples.

organs of adult female mice were examined. As can be seen in Table 5, thymic tissue and bone marrow cells showed comparable binding levels, around 50 fmoles mg^{-1} cytosol protein. In contrast, uterine tissue had 387 fmoles mg^{-1} cytosol protein, representing almost 10-fold great binding capacity than lymphoreticular tissue while a known negative tissue, esophagus, had essentially no binding activity. Binding in thymus was inhibited *in vitro* by excess (100x) zearalanol (data not shown).

The influence of estrogen on thymic hormone production was evaluated by incubating confluent cultures of thymic epithelial cells in the presence of 17β-estradiol or vehicle for 48 h. The supernatants were collected and tested for their ability to inhibit CFU-GM colony proliferation. Supernatants from thymic epithelial cells cultured in the presence of 10^{-5} M estradiol were capable of slight but significant and consistent inhibition (Table 6). In contrast, no alterations in colony formation resulted from addition of 10^{-5} M estradiol directly to the bone marrow culture, supernatants from cells cultured in the presence of 10^{-7} M estradiol or vehicle controls.

In an attempt to support the role of altered thymic epithelium factors *in vivo* on estrogen induced myelotoxicity, adult mice were surgically thymectomized (ATX) and subsequently treated with either estradiol or DES followed by evaluation of bone marrow functions. Both ATX and non-ATX mice revealed a similar degree of bone marrow hypocellularity following estrogen exposure, these values ranged from 35 to 45% below control values (Table 7). In contrast to cellularity, ATX inhibited DES and estradiol induced depression of CFU-GM and CFU-S numbers in almost all instances with all values in ATX-estrogen treated animals appearing similar to control values despite the estrogen employed or dosage administered. Subsequent studies revealed that sham-ATX mice were indistinguishable from controls with regards to estrogen myelotoxicity. Bone marrow functions in ATX estrogen-exposed mice examined 10 days following the last administration of estradiol also revealed normal CFU numbers although cellularity, in this case, was also similar to control values (Table 8). Myelotoxicity was still evident in estrogen treated intact mice.

Table 6. CFU-GM numbers following addition of culture supernatants from thymic epithelial cell monolayers (TEC) incubated with estradiol*

Supernatant added	No. of CFU-GM/10^5 nucleated cells	
	REP I	REP II
None	82 ± 1.2	60 ± 1.6
VEH (0.1% EtOH)	81 ± 2.6	64 ± 1.3
TEC	80 ± 2.4	63 ± 3.0
TEC/10^{-5}M EST	73 ± 3.0†	46 ± 1.8†
TEC/10^{-7}M EST	ND	62 ± 1.5
10^{-5}M EST‡	78 ± 2.0	58 ± 0.9

*TEC monolayers were incubated with either 0.1% EtOH (vehicle), 10^{-5} M estradiol or 10^{-7} M estradiol for 48 h. Supernatants were collected and added at a 10% v/v to methylcellulose/culture media for CFU-GM quantitation.
†$P < 0.05$ vs controls.
‡A 10^{-5} M estradiol stock solution was added to CFU-GM cultures at 10% v/v.

Table 7. Effect of adult thymectomy (ATX) on estrogen induced myelotoxicity*

ATX	Treatment	Total dosage (μmoles)	Bone marrow cellularity/femur ($\times 10^{-6}$)	CFU-GM/ 10^5 cells	CFU-S/ 5×10^4 cells
−	Vehicle	—	21.0 ± 1.2	78 ± 1	17.0 ± 0.2
−	DES	1.4	11.9 ± 0.5†	60 ± 1†	11.6 ± 0.2†
+	DES	1.4	11.8 ± 0.4†	80 ± 2	16.8 ± 0.3
−	Estradiol	2.8	15.9 ± 0.8†	ND‡	12.7 ± 0.2†
+	Estradiol	2.8	13.9 ± 0.4†	ND	15.9 ± 0.4
−	Estradiol	5.6	14.1 ± 0.9†	55 ± 2†	11.1 ± 0.3†
+	Estradiol	5.6	12.6 ± 0.7†	68 ± 2†	16.4 ± 0.4
+	Vehicle	—	19.4 ± 0.7	78 ± 1	17.2 ± 0.1
Sham	Vehicle	—	20.0 ± 0.4	ND	16.8 ± 0.2

*Mice were surgically thymectomized and treated with either DES or 17β-estradiol two days later for 5 consecutive days. Four days following the last treatment animals were tested. Each value represents the mean ± SEM of 6 mice except non-ATX vehicle which represents 12 mice.
†Significantly different from the controls at $P < 0.01$.
‡ND = not done.

Table 8. Delayed effect of ATX on estrogen induced myelotoxicity*

ATX	Treatment[a]	Bone Marrow cellularity/femur ($\times 10^{-6}$)	CFU-GM/ 10^5 Cells	CFU-S/ 5×10^4 Cells
−	Vehicle	18.5 ± 0.5	76 ± 2	17.3 ± 0.1
+	Vehicle	21.4 ± 0.6	78 ± 1	17.2 ± 0.1
−	Estradiol	14.8 ± 0.6†	63 ± 2†	12.8 ± 0.1†
+	Estradiol	17.4 ± 0.8	79 ± 1	17.1 ± 0.2

*Mice were surgically thymectomized and treated with 5.6 μmoles of 17β-estradiol as described in Table 3. Ten days following the last treatment animals were tested. Each value represents mean ± SEM of 6 mice.
†Significantly different from controls at $P < 0.01$.

DISCUSSION

While only a few studies have examined the association between estrogens and bone marrow cell function (Fried *et al.*, 1974; Boorman *et al.*, 1980; Reisher, 1966), numerous studies have addressed the occurrence of thymic atrophy in response to estrogens in animals (Dougherty 1952; Boorman *et al.*, 1980; Greenman, Dooley & Breeden, 1977; Kalland *et al.*, 1978; Bimes, DeGraeve, Amiel & Guilhen, 1975; Luz, Marques, Ayub & Correa, 1969; Sobhon & Jirasatthan, 1974). These studies suggest that thymic atrophy may be the most sensitive non-uterotrophic response of estrogens. It is clear from histological and cytological studies that increasing doses of estrogens in mice results in a progressive reduction of cortical thymocytes. Pyknosis or karyorrhexis is not a prominant feature in the atrophied thymus (Boorman *et al.*, 1980) although this point remains controversial (Sobhon & Jiresattham, 1974). Estrogens inhibit DNA synthesis in thymocytes as evidenced by reduced ^{3}H-TdR incorporation as well as reduced mitotic index (Kalland *et al.*, 1978). On the other hand, increased RNA synthesis occurs in response to estrogens in thymic epithelial cells (Thompson, Severson & Reilly, 1969) and is evidenced in histological studies where estrogens have been reported to produce cellular proliferation and hyperplasia of thymic epithelium (Plagge, 1946). In the present studies bone marrow hypocellularity and particularly depressed CFU numbers occurred at dose levels of estradiol and DES approaching therapeutic levels in humans (Morris & Van Wagner, 1966; McLachlan & Dixon, 1976). On a molar basis, DES may be slightly more effective than estradiol in causing myelotoxicity which parallels the greater *in vivo* uterotrophic activity of DES (Jacob & Morris, 1969). The observed myelotoxic effects appear not to be related to osteoproliferation which has been reported to occur following prolonged subchronic estrogen exposure (Reisher, 1966; Urist, Budy & McLean, 1950) since CFU kinetics were also altered and there was no histological evidence of estrogen-induced osteoproliferation in the present studies. In addition, these effects are not secondary to disturbances in hypothalamic control induced by ovarian dysfunction since similar effects occurred following administration of estrogen in ovariectomized mice. Other steroid hormones including testosterone and progesterone failed to affect bone marrow functions providing evidence of an estrogen-specific phenomenon. Testosterone, which has slight estrogenic activity when given at high doses (Schmidt, Sadler & Katzenellenbogen, 1976), is structurally similar to estradiol in the D-ring region but not A-ring region of the molecule. The phenolic A ring structure is responsible for receptor binding activity and specificity of estrogenic compounds (Duax & Weeks, 1980). Testosterone, while having little if any effect on lymphoid or myeloid precursor cells has previously been shown to be an effective stimulant rather than depressant of erythropoiesis (Hogen & Cody, 1972; Erslav, 1962).

Metabolic studies have led to the identification of oxidative metabolites for both DES and estradiol (Metzler, Gottschlich & McLachlan, 1980). The most abundant DES metabolite is Z,Z-DIES which is of particular interest since it is not estrogenic but demonstrates marked genotoxicity in sister chromatid exchange assays and is capable of binding to macromolecular substances (Rudiger, Haenisch, Metzler & Glatt, 1979) and for these reasons may be a potential carcinogenic metabolite. However, when mice were administered relatively high levels of Z,Z-DIES, no myelotoxicity or thymic atrophy were observed. On the other hand, myelotoxicity and thymic atrophy were obtained following exposure to the estrogenic dienestrol isomer, E,E-DIES, considered to be a DES analog. These results indicate an epigenetic mode of action consistant with hormonal activity. The data, however, does not exclude the possibility that metabolites that possess nonspecific

membrane reactivity may ultimately be responsible for myelotoxicity.

The relationship between myelotoxicity and estrogenic activity was further examined by employing estrogen antagonists. Two classes of non-steroidal antiestrogens in the rat uterus have been identified and include derivatives of triphenylethylene and diphenyl (dihydro or tetrahydro) napthalene, the most notable representatives of these classes being Tamoxifen and Nafoxidine, respectively (Sutherland & Murphy, 1982). Although these compounds undergo nuclear accumulation and have limited estrogenic activity, they appear to inhibit replenishment of cytoplasmic receptors. Thus, they act as partial antagonist in the rat uterus following subsequent estrogen injection (Clark, Watson, Upchurch, McCormack, Padykula, Markaverich & Hardin, 1980). The reduced thymic atrophy that occurred in antiestrogen treated mice or estrogen treated mice pretreated with antiestrogen, although not remarkable, is consistent with the observed agonistic properties of these compounds in the uterus. However, administration of antiestrogens caused almost complete inhibition of estrogen-induced myelotoxicity. While the mechanism for this profound antagonism in the bone marrow, rather than partial antagonism as seen in the thymus is unknown, it would appear that the initial events associated with myelotoxicity, whether direct or indirect, may be mediated through a receptor-like mechanism.

It is now known that most estrogenic activity is initiated following specific binding to cytoplasmic receptors and subsequent translocation into the nucleus (Korach, 1979; Clark, *et al.*, 1980; Korach, Metzler & McLachlan, 1978). In rodents, cytosol prepared from the thymus has been reported to contain a binding molecule which has many similar characteristics to the estrogen receptor in the uterus (Gillette & Gillette, 1979; Grossman, Sholiton & Nathen, 1979) and more recently has been found to translocate to the nucleus of thymus preparations in the form of a receptor-ligand complex (Screpanti, Grutino & Pasqualini, 1982). The present studies confirm the presence of an estrogen-binding component present in mouse thymic cytosol. Furthermore, we demonstrate potentially significant but not high binding levels in the cytosol fraction of bone marrow cells. The biological significance of these components in lymphoreticular tissue is presently unknown but may be involved in the initial myelotoxic events. This was suggested by the fact that pretreatment of mice with antiestrogens, which presumably exert their antagonistic properties by utilizing the estrogen receptor (Sutherland & Murphy, 1982; Clark *et al.*, 1980) prevent subsequent estrogen induced myelotoxicity. These data, however, should be interpreted with some caution particularly since relatively high levels of the estrogen antagonists were employed to examine potential antiestrogenic activity. In addition, extensive studies with glucocorticoid receptors present in the cytosol of lymphocytes have failed to clearly establish a role for the receptor in corticosteroid induced immunosuppression (Cupps & Fauci, 1982).

Estrogens have been reported to induce adrenal cortical hypertrophy via anterior pituitary activation (Heywood, Lewis & Wadsworth, 1980) as well as alter adrenomedullary catecholamine secretion (Wiechman & Borowitz, 1979). Furthermore, corticosteroids alter the bone marrow environment (Bennett, 1970; Cohen & Claman, 1971). Studies with adrenalectomized mice, however, clearly indicate that estrogen-induced myelotoxicity and thymic atrophy are not related to altered adrenal function. Adrenalectomy has also been shown not to compromise estrogen stimulation of other target tissues such as the uterus or vagina (Quarmby, Fox-Davies, Swaisgood & Korach, 1982). Thus, it would appear that the adrenal pathology (cytoplasmic vacuola) observed in estrogen treated mice was coincidental to the thymic atrophy and myelotoxicity that occurred following estrogen exposure.

Several lines of evidence suggest that estrogen-induced myelotoxicity, at least in part, is mediated through the thymus, particularly at the level of regulation of CFU kinetics. Additional events may be responsible for the occurrence of hypocellularity. This bimodal effect was first recognized in thymectomy experiments where thymic abolution prevented progenitor cell inhibition but had no effect on estrogen induced marrow hypocellularity even at doses up to 5.6 μmoles of estradiol. Thymic involvement in the regulation of hematopoiesis has been well recognized although whether these events are mediated through cell – cell recognition, release of active factors from the thymus or products of T-lymphocytes remains controversial (Goodman, Basford & Shinpock, 1978; Goodman, Shinpock & Basford, 1979; Burek, Plavljanic, Slamberger & Vitale, 1977; Hamano & Nagai, 1978; Lepaul, Dardenne & Frindel, 1979; Zipori & Trainin, 1975). There is growing evidence, however, that thymic factors are necessary to initiate DNA synthesis (Lepault *et al.*, 1979; Zipori & Trainin, 1975) in normally quiescent medullary CFU-S (Becker, McCulloch, Siminovitch & Till, 1965) and that ATX inhibits the ability of various stimuli such as bleeding or antigenic stimulation to initiate CFU DNA

synthesis (Lepault *et al.*, 1979; Frindel & Croizat, 1975). This would suggest that stem cells in S-phase of cell cycle are preferentially susceptible to the thymic regulatory factor(s). Of particular significance to the present studies is the identification of a factor recently observed in sera of estrogen treated rats, but not estrogen exposed-ATX rats, which suppresses lectin induced lymphocyte blastogenesis (Stimson & Hunter, 1980). Preliminary studies shown here demonstrate the existence of a factor present in supernatants of mouse thymic epithelial cells cultured in the presence of 10^{-5} M 17β-estradiol which inhibits CFU-GM colony growth. This suggests that estrogens alter CFU kinetics, in part, by altering production of regulatory products from the thymic epithelium. Further evidence that this represents a "negative" regulatory factor of thymic origin was evidenced by the ability of ATX to protect against depression of CFU kinetics. The fact that supernatants from TEC incubated with 10^{-7} M estradiol failed to produce this inhibitory factor(s) would suggest that this is not a receptor mediated event, although chemical availability was not determined. When gradient purified thymocytes were substituted in culture for thymic epithelial cells, no inhibitory effects were detectable, providing additional evidence that this is a thymic epithelial product and not a lymphokine (data not shown). In any case, further studies will be required to determine the exact nature of this inhibitory factor.

Ten days following cessation of estrogen exposure marrow hypocellularity and CFU depression were still evident in intact estrogen exposed mice (see Table 4). Furthermore, "ATX-protection" of CFU-kinetics was still present. Of interest was the rapid repopulation of marrow to normal cellularity in ATX-estrogen treated mice which is not protected following ATX-estrogen exposure. This was predictable since there was no loss of progenitor cells which would allow for repopulation to occur.

That other mechanisms are involved in estrogen myelotoxicity other than altered thymic regulation was suggested in studies with zearalanol. Zearalanol is an estrogenic metabolite from the mycotoxin zearalanone, which is produced from various species of *Fusarium* and has been associated with an estrogenizing syndrome in cattle fed mold-infected grain (Pathre & Mirocha, 1980). Zearalanol was of interest to study because it contains ~20% of the binding affinity of estradiol but only 1% of its uterotrophic activity (Katzenellenbogen, Katzenellenbogen & Mordecai, 1978). Zearalanol was almost as effective as DES and estradiol, on a molar basis, in inhibiting CFU-S and CFU-GM proliferation, however, cellularity was not affected. While this may be a reflection of dose-response, it does indicate that these events are probably mediated by different mechanisms. Similarly, estrogen induced hypocellularity appears to be less sensitive to the influence of estrogens than progenitor cell depression since CFU numbers were decreased after a single dose of 0.56 μmoles of DES without evidence of hypocellularity (data not shown). Thus, the hypocellularity may not be at the level of thymic regulation and may simply reflect mobilization of the marrow cells into other body compartments. In this respect estrogens cause marked increases in circulating leukocyte values and resident peritoneal cell numbers as well as splenomegaly (Boorman *et al.*, 1980).

In summary, pharmacological levels of estrogens profoundly influence bone marrow functions in mice. Estrogen-induced alterations in regulatory factors produced by thymic epithelial cells appear, at least in part, responsible for reduced CFU kinetics. In addition, estrogens may also influence bone marrow function through non-thymic mechanisms as evidenced by hypocellularity which occurs independent of thymic influences. There is suggestive data that these events, particularly the former, are mediated through estrogen receptor or receptor-like mechanisms.

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