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SUPRAPHYSIOLOGICAL LEVEL OF ESTROGEN EXPOSURE IN VIVO INCREASES LYMPHOID CELL DEATH IN MICE

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Summary

Estrogen can enhance or reduce lymphocyte functions in vitro depending on dose and exposure duration. The purpose of this study was to determine the effect of in vivo 17β-estradiol (E2) on apoptosis and necrosis in lymphoid tissue of female C567BL/6 mice. Animals were ovariectomized (OVX), ovariectomized and 17β-estradiol supplemented (OVX+E2; 71 μg E2 per day for 14 days), sham ovariectomized (SHAM), or unhandled controls (CONTROL). Thymus and spleen were removed aseptically, cells dispersed into single cell suspensions in RPMI-1640, and measures of cell damage performed: an annexin V flow cytometric assay for markers of apoptosis and an enzyme-linked immunoassay for measures of DNA fragmentation and necrosis. OVX+E2 mice had 620 ± 72 pg/ml 17β-estradiol in serum in contrast to OVX mice which had 7.6 ± 5 pg/ml, the SHAM mice which had 2.8 ± 1 pg/ml of serum E2, and the CONTROL mice which had 3.9 ± 0.8 pg/ml of serum E2 ($p<0.001$). There was a significantly lower percentage of viable thymocytes in OVX+E2 mice compared to the other treatment conditions ($p<0.001$, respectively). There was also a significantly higher percentage of annexin V positive thymocytes in OVX+E2 mice ($p<0.005$). Measures of DNA fragmentation by ELISA were higher in splenocytes from OVX+E2 mice than in the OVX, SHAM or CONTROL mice ($p<0.005$). These results suggest that supraphysiological levels of estrogen in vivo induce damage in lymphoid cells; however, the impact of estrogen associated lymphoid tissue damage on specific immune functions remains to be determined.

Key Words: estrogen, ovariectomy, apoptosis, thymus, spleen

There is a growing body of evidence that estrogens regulate immune responses and may contribute to the etiology of immune-mediated diseases. The estrogen link is particularly suggestive for the pathogenesis of certain autoimmune diseases (1-3). For example, the incidence of systemic lupus erythematosus (SLE) is higher in premenopausal compared to postmenopausal women (4). That

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estrogens influence a variety of immune functions have been studied using in vitro models by a number of investigators. Sorachi et al. (5) found an enhancement in the in vitro natural killer cell cytotoxicity and T lymphocyte proliferation following treatment with estradiol. In contrast, Baral and colleagues (6) found that estrogen significantly decreased natural killer cell cytotoxicity against tumor targets but this effect was dependent on duration of exposure to estrogen. Reduced natural cytotoxicity as a result of estrogen exposure has also been reported (7); estrogen significantly suppressed natural killer cell lysis in a dose-dependent fashion and this was hypothesized to be due to impaired peroxidase or oxygenase function. Estrogens are potent regulators of the expression of proinflammatory cytokines, such as tumor necrosis factor and interleukin-6, from cells of the immune system (8); moreover, several of the proinflammatory cytokines play a role in regulating apoptosis (9).

The purpose of this study was to determine if estrogen was associated with the induction of apoptosis in cells of the immune system. We examined two measures of cell damage and death in the thymus and spleen following in vivo exposure to supra-physiological levels of estrogen in female ovariectomized mice. The data indicate that high levels of estrogen exposure in vivo enhance apoptosis and cell death in primary and secondary immune compartments.

Methods

Experimental Animals. Female C57BL/6 mice (Harlan Sprague-Dawley, Indianapolis, IN), age 10-11 weeks, were randomly assigned to the following conditions: ovariectomy (OVX), ovariectomy and 17β-estradiol pellet supplement (OVX+E2) which released 71.4 μg E2 per day over 21 days, surgical sham with placebo pellet (SHAM), or unhandled controls (CONTROL). Estrogen and placebo pellets were obtained from Innovative Research Corporation of America (Sarasota, Florida). The mice were housed in groups of 4 animals in standard cages, with inclusion of nesting material and a short length of PVC tubing for environmental enrichment. The mice were housed in a vivarium maintained at 21± 2°C on a 12/12 h reversed light/dark cycle. Food (rodent chow, PMI Feeds, Richmond, IN) and tap water were provided ad libitum. All procedures involving mice were conducted in accordance with guidelines established by the Canadian Council on Animal Care.

Ovariectomy. Mice were anaesthetized with Avertin (tribromoethnaol, 0.016 per g body wt), a midline dorsal incision was made in the lumbar region to reveal the dorsal fat pads containing the ovaries, the ovaries were pinched off, and mice were implanted with either estrogen or placebo pellets. Mice were given temsgesic (subcutaneous, 0.05-0.1 mg/kg), recovered on a heating pad, and returned to home cages for 14 days at which time they were sacrificed for lymphoid tissues.

Tissue Collection. Blood was removed by cardiac exsangination, centrifuged at 500 x g, and plasma stored at -20°C until use. Thymus and spleen were removed aseptically, weighed, and placed in small culture dishes containing RPMI-1640 with 10% fetal bovine serum. Single cell suspensions of the tissues were prepared by gentle passage through a fine, sterile nytox mesh. The suspensions were layered onto a Percoll density gradient (Lympholyte-M®, Cedarlane Laboratories, Hornby, ON), centrifuged at 1000 x g for 20 min, lymphocytes at the interface rapidly collected and washed in sterile medium, and final cell concentrations adjusted to $1x10^6$ cell/ml in 4 ml volumes of phenol-red free RPMI-1640.

Measurement of Apoptosis by Flow Cytometry. Measurement of apoptotic, necrotic, and viable thymocytes and splenocytes was determined using annexin V FITC and propidium iodide (Coulter-Immunotech, France) as previously described (10). This method assays changes in the expression of phosphatidylserine on cell surfaces during the early stages of apoptosis.

Measurement of Cell Death by ELISA. Cell death (apoptosis and necrosis) was assessed using a commercially available enzyme-linked immunoassay kit (Boehringer-Mannheim, Canada) which determines the amount of cytoplasmic histone-associated DNA fragments. The detected absorbance is reflective of the DNA fragments present in the sample, as absorbance increases with the number of fragments present. In brief, 10 µl (~ 1000 cells) from each sample cell suspension was diluted in 100 µl of sterile PBS, spun down to pellet, and resuspended in a supplied lysis buffer. Samples were incubated 30 min at room temperature, centrifuged at 200 x g, and mono- and oligonucleosomes collected from the cell lysate. Samples were seeded into 96well microtitre plates, and 80 µl of incubation solution (anti-DNA antibodies, anti-histone antibodies, incubation buffer) was added to each well. The microplate was covered, incubated at room temperature for 2 h on a plate shaker, following which the sample wells were rinsed gently 3 times in 200 µl of incubation buffer. To each sample well, 100 µl of stock colorimetric solution (ABTS tablet in 15 ml of substrate buffer) was added, the plate was covered and incubated at room temperature for 10-15 min, and the samples were read on a microplate reader at a wavelength of 405 nm.

Plasma Estradiol. Plasma 17β-estradiol, obtained from a subsample of mice at the time of sacrifice, was measured using a commercially available ^{125}-I double antibody radioimmunoassay kit (Inter-Medico). Estradiol was first extracted under diethyl ether. The inter-assay coefficient of variation (CV) and intra-assay CV for this kit are reported to be 4.2-8.1% and 4.0-7.0%, respectively. The detection limit at 95% concentration is 8 pg/ml and the cross-reactivity with other estrogenic compounds was negligible.

Statistics. Statistical analyses were performed using SAS (SAS 6.12, SAS Institute Inc., Cary, NC). One way analysis of variance were performed for the main effect of hormone for the measures of lymphoid tissue apoptosis/cell damage and for plasma estradiol. If significant effects were found, post-hoc analyses were carried to determine the specific group differences using Tukey's post-hoc test. A value of $p < 0.05$ was considered to be significantly different from that observed from chance alone. All values are group means ± one standard error.

Results

Plasma Estradiol Levels. Table I shows the mean plasma estradiol values for the treatment conditions. As expected there was a significant effect of hormone supplementation on the plasma estradiol levels ($p<0.0001$). Mice that received the estradiol supplement had significantly higher plasma estradiol concentrations compared to the other treatment conditions.

Thymus and Spleen Cellularity. Table II shows the effect of hormone supplementation on thymic and splenic tissue cellularity. There was a significant effect of hormone treatment on thymic and splenic cellularity ($p<0.0001$ and $p< 0.0001$, respectively). OVX+E2 supplemented mice had significantly lower cell counts relative to the other hormone conditions.

TABLE I

Effect of Ovariectomy and Hormone Replacement on Plasma Estradiol Concentrations in Mice

Treatment Group	Plasma Estradiol (pg/ml) (Mean ±S.E.M.)	Sample Size
CONTROL	3.9 ± 0.73^a	5
SHAM	2.8 ± 1.1^a	5
OVX	7.6 ± 5.4^a	6
OVX + E2	620.7 ± 71.7^b	6

Superscripts that differ indicate significant effects at $p<0.05$ (Tukey's post-hoc test).

TABLE II

Effect of Ovariectomy and Hormone Replacement on Murine Thymic and Splenic Cellularity

Experimental Group (n)	Mean Thymus Count (10^6) ± 1 SEM	Mean Splenocyte Count (10^6) ± 1 SEM
CONTROL (12)	56.4 ± 7.2^a	33.9 ± 3.8^a
SHAM (7)	52.7 ± 10.7^a	34.5 ± 6.6^a
OVX (24)	79.0 ± 8.1^a	35.2 ± 3.1^a
OVX+E2 (21)	4.7 ± 0.9^b	13.5 ± 1.1^b

For thymus and spleen cell counts, superscripts that differ indicate significant effects at $p<0.05$ (Tukey's post-hoc test).

Annexin V Measures of Apoptosis in Thymus and Spleen. Fig. 1 shows the percentage of apoptotic, necrotic, and viable cells in the thymus of ovariectomized mice, with and without estradiol supplementation. The percentage of apoptotic thymocytes and viable thymocytes differed significantly as a function of hormone condition ($p<0.005$ and $p<0.001$, respectively). These differences were due to the higher percentage of apoptotic thymocytes and lower percentage of viable thymocytes in OVX+E2 supplemented mice relative to OVX mice.

Fig. 2 shows the percentage of apoptotic, necrotic, and viable cells in the spleen of ovariectomized mice, with and without estradiol supplementation. The percentage of viable splenocytes was marginally but significantly lower in OVX+E2 supplemented mice relative to the OVX mice ($p<0.05$). There were no significant hormone effects on the percentage of apoptotic and necrotic splenocytes.

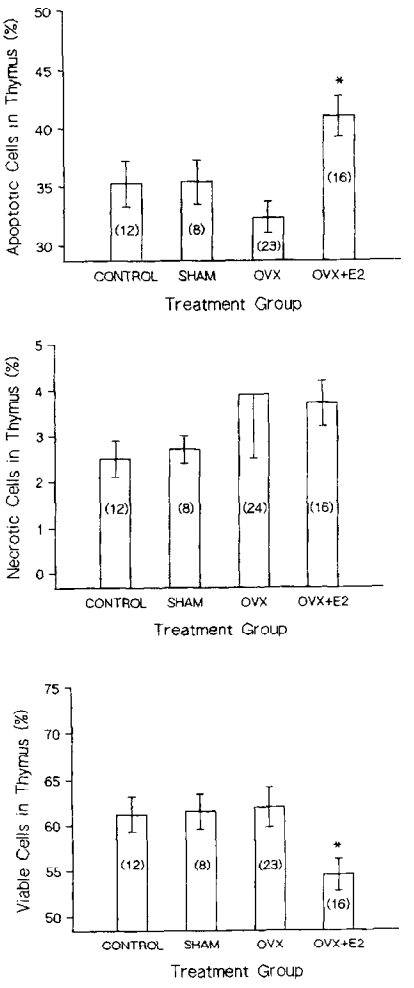

Fig. 1

Effect of ovariectomy and estradiol replacement on the percent of apoptotic, necrotic and viable thymocytes from C57BL/6 mice. * significant at $p<0.05$ vs. OVX. Numbers in parenthesis are sample sizes.

Cell Death ELISA. Fig. 3 shows the mean absorbance values (indicative of degree of fragmentation of nuclear material) by hormone treatment for thymocytes obtained from C57BL/6 mice (top panel). There was no significant difference in the absorbance values as a function of hormone treatment, although OVX+E2 tended to have higher values (i.e., greater DNA fragmentation) than the other groups. Fig. 3 (bottom panel) shows the mean absorbance values by hormone treatment for splenocytes obtained from mice. There was a significant main effect of hormone supplementation on DNA fragmentation ($p<0.005$), with mice in the OVX+E2 group having higher absorbance values compared with the OVX group.

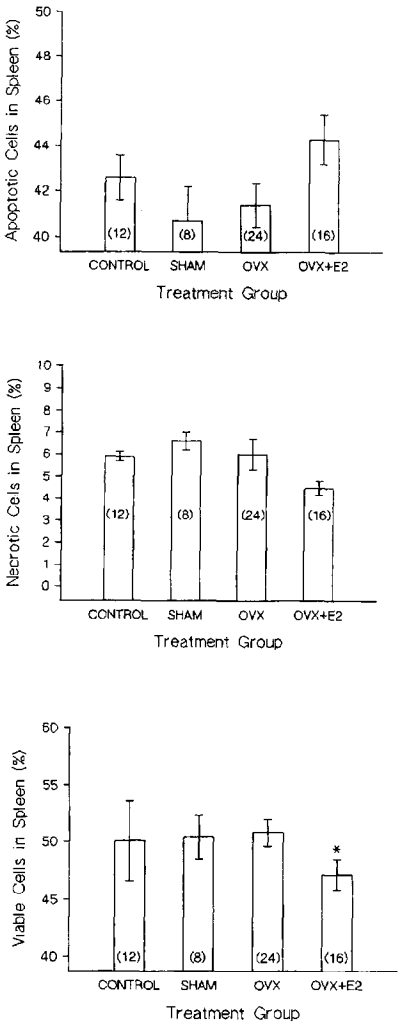

Fig. 2

Effect of ovariectomy and estradiol replacement on the percent of apoptotic, necrotic and viable splenocytes from C57BL/6 mice. * significant at $p<0.05$ vs. OVX. Numbers in parenthesis are sample sizes.

Discussion

Not surprisingly, ovariectomized mice replaced with high doses of exogenous 17β-estradiol had significantly higher levels of free hormone compared to the control mice and to those which were ovariectomized but not replaced with hormone. That the levels of plasma 17β-estradiol did not differ between controls and ovariectomized mice was not unexpected; in ovariectomized mice, there continues to be a residual peripheral conversion of androgenic compounds to estrogens through enzymatic pathways present in adipose tissue and, further, there is a natural production of androgenic compounds in the adrenal glands. Minimal levels of plasma hormone in control (surgical sham and

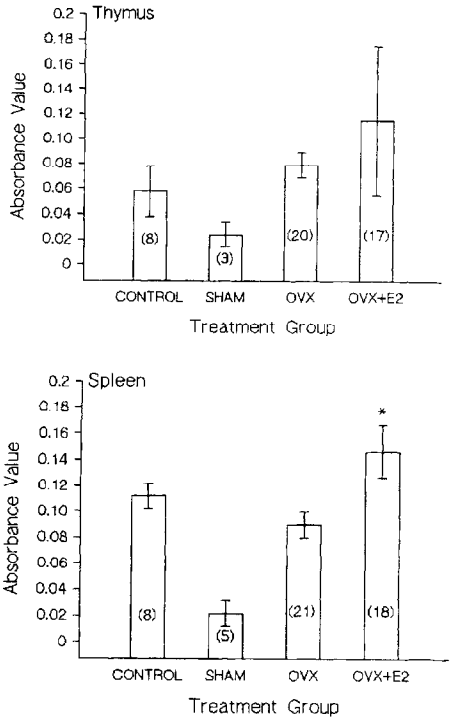

Fig. 3

Effect of ovariectomy and estradiol replacement on cell death ELISA absorbance values from thymus (top panel) and spleen (bottom panel) of C57BL/6 mice. * significant at $p<0.05$ vs. OVX. Numbers in parenthesis are sample sizes.

cage controls) were also not unexpected. When female mice are housed in groups without the influence of a male mouse, induction of a state of pseudo pregnancy occurs (11). In this state, blood levels of estrogenic compounds drop to extremely low levels (12) similar to those observed in this study. The values obtained in the control and ovariectomized mice were within the reported physiological range of mouse estradiol levels of 5-50 pg/ml (12).

The results of this study show significantly fewer viable splenocytes from OVX+E2 replaced mice compared to the OVX condition. These findings suggest a shift away from a viable cell population to cell damage, even if not immediately evident in the corresponding number of overtly apoptotic or necrotic cells. Thymic samples were also suggestive that high levels of estradiol supplementation induce cell damage as determined by the percentage of apoptotic thymocytes relative to unsupplemented and control mice. The differences between the two compartments (thymus, spleen) in response to estradiol replacement may reflect the different cell populations. The population of the spleen is made up of peripheral, mature lymphocytes whereas the thymus consists of T-lymphocytes in various stages of maturation. The susceptibility of immature thymocytes and transformed T-lymphocyte cell lines to induction of apoptosis in response to various stimuli has been well established. Mature lymphocytes show greater resistance to apoptotic events. For example, Wesselborg et al. (13) examined the ability of mature peripheral T-lymphocytes to respond to

various stimulant agents (e.g., OKT3, an anti-CD3+ antibody, and PHA, a mitogen). Although freshly obtained T-lymphocytes were resistant to apoptosis, there was a gradual acquisition of susceptibility upon repeated mitogenic stimulation and extended culture. The findings of Wesselborg and colleagues suggest that mature T-lymphocytes are inducible to apoptosis when in an activation state but show greater resistance in a resting, unstimulated state. The measures of cell death ascertained by ELISA also suggested that high levels of estrogen supplementation increase the likelihood of cell damage and DNA fragmentation in lymphoid tissue compartments. OVX+E2 mice had significantly more DNA fragmentation in splenocytes and tended to have greater cell damage in thymocytes compared with unsupplemented animals. Nevertheless, the thymus samples taken from OVX+E2 mice showed extremely large variability (Figure 3), obscuring any group treatment effects. The reasons for this variability are not known but may reflect the individual responses to a pharmacologic dose of estrogen.

The effect of estrogen on cells of the immune system has been studied extensively, with often contradictory findings (14, 15). Less is known about the effects of estrogens on the induction of apoptosis in lymphoid cells. Subbiah and colleagues (16) examined a variety of estrogen preparations used in postmenopausal HRT. 17β-estradiol enhanced the oxidative potential of activated macrophages and this effect was blocked with equilenin (an unsaturated equine estrogen). Forsberg (17) found that adult female mice treated with diethylstilbestrol had thymus weights significantly lower than controls and variable thymolytic patterns depending on time of sampling. Evans et al. (4) reported that physiological levels of estrogen reduced the rate of in vitro apoptosis of peripheral blood mononuclear cells (PBMC) of premenopausal women; however, among noncycling, postmenopausal women addition of estrogen to PBMC culture increased apoptosis, The mechanisms for these apoptosis promoting effects in lymphoid cells have been suggested to include modulation of prostaglandin synthesis (18), tumor necrosis factor production (14), or interferon-gamma production (19).

There was a marked reduction in thymic and splenic cellularity in OVX+E2 replaced mice. These findings are consistent with the published literature (20,21,22) showing that removal of sex hormones is associated with increased splenic and thymic cellularity while supplementation with sex hormones can reverse this effect.

In conclusion, our data indicate that ovariectomy with replacement of supra-physiological levels of estrogen increases the induction of apoptosis and cell death in thymic and splenic lymphoid cells of female mice. Additionally, estrogen supplemented animals had marked reductions in the cellularity of these primary and secondary lymphoid tissues. The clinical implications of the greater apoptosis and cell death in estrogen supplemented animals are yet to be determined. However, given that HRT selectively reduces the percentage and function of circulating immune cytotoxic effector cells (23), it is possible that apoptotic mechanisms contribute to altered immunity observed in postmenopausal women on estrogen replacement therapy. An important caveat of this study was the supraphysiologic (or near pharmacologic) level of estrogen that was used in vivo. Further research is needed to determine the dose-response characteristics to estrogen of thymocyte and splenocyte apoptosis and necrosis, and the implications for postmenopausal women using HRT.

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References

1. D. BOUMPAS, B. FESSLER, H. AUSTIN, J. BALOW, J. KLIPPEL, M. LOCKSHIN. Ann. Intern. Med. **123** 42-53 (1995).
2. M. CUTOLO, A. SULLI, B. SERIOLO, S. ACCARDO, A. MASI. Clin. Exp. Rheum. **12** 217-226 (1995).
3. J. SANCHEZ-GUERRERO, M. LIANG, E. KARLSON, D. HUNTER, G. COLDITZ. Ann. Intern. Med. **122** 430-433 (1995).
4. M.J. EVANS, S.MacLAUGHLIN, R.D. MARVIN, N.I. ABDOU. Clin. Immunol. Immunopathol. **82** 258-262 (1997).
5. K. SORACHI, S. KUMAGAI, M. SUGITA, J. YODOI, H. IMURA. Immunol. Letts **36** 31-36 (1993).
6. E. BARAL, E. NAGY, I. BERCZI. Cancer **75** 591-599 (1995).
7. M. FERGUSON, F. McDONALD. FEBS **191** 145-148 (1985).
8. S.H. ZUCKERMAN, S.E. AHMARI, N. BRYAN-POOLE, G.F. EVANS, L. SHORT, A.L. GLASEBROOK. Inflammation **20** 581-597.
9. F. NOVELLI, F. DI PIERRO, P.F. DI CELLE, S. BERTINI, P. AFFATICATI, G. GAROTTA, G. FORNI. J. Immunol. **152** 496-504 (1994).
10. L. HOFFMAN-GOETZ, S. ZAJCHOWSKI, A. ALDRED. Life Sci. **64** 191-200 (1999).
11. K. RYAN, N. SCHWARTZ. Biol. Reprod. **17** 578 (1977).
12. K. RYAN, N. SCHWARTZ. Endocrinol. **106** 959-966 (1980).
13. S. WESSELBORG, O. JANSSEN, D. KABELITZ. J. Immunol. **150** 4338-4345 (1993).
14. T.-C. CHAO, P.J. VAN ALTEN, J.A. GREAGER, R.J. WALTER. Cell. Immunol. **160** 43-49 (1995).
15. I. SCREPANTI, A. SANTONI, A. GULINO, R. HERBERMAN, L. FRATI. Cell. Immunol. **106** 191-202 (1987).
16. M. SUBBIAH, B. KESSEL, M. AGRAWAL, R. RAJAN, W. ABPLANALP, Z. RYMASZEWSKI. J. Clin. Endocrinol. Metab. **77** 1095-1097 (1993).
17. J.G. FORSBERG. Acta Anat. **157** 275-290 (1996).
18. C.F..ROSENKRANS, JR., B.C. PARIA, D.L. DAVIS, G. MILLIKEN. Prostaglandins **43** 309-315 (1992).
19. H.S. FOX, B.L. BOND, T.G. FARSLOW. J. Immunol. **146** 4362- (1991).
20. D.N. KALU, E. SALERNO, C. LIU, F. FERARRO, B. ARJMANDI, M. SALIH. Bone Miner. **23** 145-161 (1993).
21. D. EIDINGER, T. GARRETT. J. Exp. Med. **136** 1098- 1116 (1972).
22. H. CHIODI. Endocrinol. **26** 107-116 (1940).
23. R. BRUNELLI, D. FRASCA, G. PERRONE, C. PIOLI, A. FATTOROSSI, L. ZICHELLA, G. DORIA. Gynecol. Obstet. Invest. **41** 128-131 (1996).