

Effects of Xenoestrogens on T Lymphocytes: Modulation of bcl-2, p53, and Apoptosis

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Abstract: Endogenous estrogens have significant immunomodulatory effects characterized as suppression of cell mediated immunity and stimulation of humoral immunity. Xenoestrogens are environmental estrogens that have endocrine impact, acting as estrogen agonists and antagonists but whose immune effects are not well characterized. Using CD4⁺ Jurkat T cells as a model, the effects of representative xenoestrogens on T proliferation, cell cycle, and apoptosis were examined. Coumestrol (CM), a phytoestrogen, and tetrachlorodioxin (TCDD) in concentrations of 10⁻⁴ to 10⁻⁶M significantly inhibited Jurkat T cell lymphoproliferation, whereas bisphenol A (BPA) and DDT had minimal effect, but did antagonize 17-β-estradiol induced effects. Xenoestrogens, especially CM, produced accumulation of Jurkat T cells in G₂/M phase, and subsequently induced apoptosis, particularly CM (% apoptotic cells = 30 ± 12 vs. control = 5 ± 2). These changes were associated with DNA fragmentation. BPA and DDT also induced DNA fragmentation but not significant DNA hypoploidy. Xenoestrogen – CM, BPA, DDT, and TCDD - exposure suppressed bcl-2 protein and mRNA transcript levels but augmented p53 protein and mRNA transcripts. Human purified peripheral blood lymphocytes responded with similar significant cell cycle changes (G₀/G₁ exodus and G₂/M accumulation) for CM, BPA, and DDT exposure. These preliminary data, taken together, suggest that xenoestrogens have direct, compound-specific T lymphocyte effects that enhance our understanding of environmental modulation of immune and autoimmune responses.

Keywords: Xenoestrogens, coumestrol, bisphenol A, DDT, TCDD, T lymphocytes, cell cycle.

1. Introduction

Endogenous estrogens, especially 17- β -estradiol, have significant immunoendocrine impact on cell-mediated and humoral immune and autoimmune responses [1-5]. Derived from plant or industrial synthesis, environmental xenobiotics with potential estrogenic or hormonal activities are known as xenoestrogens or ecohormones. These compounds are ubiquitous, exhibit bioaccumulation, and act as estrogen agonists or antagonists, disrupting normal endocrine axes [6-15]. Xenoestrogens have significantly weaker binding affinities than endogenous estrogens to traditional steroid receptors [9, 14,15] and their medical, environmental, and societal impact is the frequent subject of debate [12,13]. Nevertheless, xenoestrogen impact on lymphocyte, inclusive of investigations of 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and genistein, appears incompletely characterized.

Representative xenoestrogens include compounds such as coumestrol, a phytoestrogen found in high levels in legumes that acts as an estrogen agonist. Coumestrol induces T cell chromosomal abnormalities [16] and modulates production of thymic hormones [17]. Bisphenol A, a polycarbonate monomer associated with plastics and dental compounds, also has estrogenic agonist/antagonist effects, has been associated with advancement of puberty, and appears to suppress lymphocyte mitogenesis [18-20]. DDT (o,p-dichlorodiphenyltrichloroethane), a synthetic organochlorine pesticide, has weak estrogenic agonist activity (as well as androgen antagonist activity) [21] and has been associated with immunosuppression in murine models [22-24] and modulation of cell cycle and apoptosis [25-27], but its effects on T lymphocyte biology is less well defined. TCDD (tetrachlorodibenzo-p-dioxin), a polychlorinated biphenyl dioxin, has been widely studied with variably results, having both estrogen agonist and antagonist activity [6,14,21], modulation of cell cycle proteins [28,29], induction of thymic involution [30], and modulation of cytokine expression [31,32].

Xenoestrogens may act at the cellular and molecular levels, binding to both steroid and aryl hydrocarbon receptors exhibiting both dependent and independent receptor modulations of specific gene transcriptional elements [29,32-35]. As a result, ecohormones have the potential to variably modulate lymphocyte proliferation, cell cycle progression, apoptosis and cytokine production in much the same way as 17- β -estradiol does [36-40]. This modulation is likely to occur in association with alterations in T lymphocyte bcl-2 or p53 protein levels [28,29,38,39]. In this investigation, using the CD4⁺ Jurkat T lymphocyte cell line as a model [38-40], xenoestrogen-specific effects on T lymphocyte biology were initiated.

2. Material and Methods

2.1. Reagents and Cell Culture

Phenol-red free RPMI and media supplements were purchased from Gibco/BRL (Gaithersburg, MD). Xenoestrogens (coumestrol - CM, bisphenol A - BPA, DDT and TCDD), fetal calf sera, and

phorbol-myristate acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-CD3 antibody was obtained from Becton-Dickinson (CA). The CD4⁺ Jurkat T cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in logarithmic growth in phenol-red-free RPMI 1640 supplemented with 5% charcoal stripped (steroid free) FCS and 200 mM glutamine, and 1% penicillin/streptomycin. Steroid-free serum was utilized to exclude confounding effects from steroids in the supplemented media. Peripheral blood T cells were obtained by venipuncture from healthy volunteers after informed consent and were isolated from peripheral blood through Ficoll-Hypaque (Sigma, St. Louis, MO) centrifugation and removal of adherent cells by plastic adherence in 20% FCS. Flow microfluorometric analysis revealed 95-97% T cell purity (data not shown). After thorough washing, Jurkat or peripheral blood T cells were cultured at 1×10^6 cells/ml in 24 well plates. As indicated, T cells were activated with PMA and plate-bound anti-CD3 at optimal concentrations as previously described [40]. 17- β -estradiol and TCDD were used as representative controls of endogenous and environmental estrogen compounds unless otherwise indicated.

2.2. Lymphoproliferation

Cells were cultured in triplicate at 0.5 or 1.0×10^6 /ml for up to 72 hours. Proliferation was measured by determination of total viable cell mass using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. Absorbance at 490 nm was determined on a Bio-Rad (Hercules, CA) plate reader.

2.3. Cell Cycle Analysis

Cell cycle analysis was performed as previously described [38,39]. Briefly, 1×10^6 were grown in suspension, harvested by centrifugation, washed, and fixed in 1% paraformaldehyde. After washing, the cells were permeabilized in 70 % ethanol, washed, and re-suspended in PBS. RNase (Sigma) was added to a final concentration of 5 U/ml. Cells were stained with PI, flow microfluorometry was performed, and DNA histograms were generated and analyzed using a Becton Dickinson Flowscan (Franklin Lakes, NJ). This method correlates closely with other measures of apoptosis including TUNEL and Annexin V staining while providing additional cell cycle information [41]. This method also allows for enumeration of the percentages of cells in G₀/G₁ (resting phase), S (DNA synthesis phase), G₂M (mitotic phase), and hypodiploid or apoptotic percentages (those cells containing less than the normal amount of DNA) [41].

2.4. DNA Fragmentation

Examination of apoptosis by electrophoresis of nucleosomal fragments was performed by a modified procedure precipitating cytosolic nucleic acid as previously described [38]. Results were identical to those in which total DNA is prepared but this modification allows for easier resuspension of the DNA. Fifteen μ l was electrophoresed on a 1.8 % agarose gel and stained with ethidium for visualization.

2.5. Western blot analysis

For Western analysis, 2 to 5×10^6 cells were cultured (1.0×10^6 /ml) and stimulated bcl-2 and p53 protein levels were assayed as previously described [38,39]. Total protein concentration determined by the method of Bradford and Lowry using Bio-Rad Protein Assay reagents (Bio-Rad) in a microtiter assay plate. Thirty μ g of total cellular protein was electrophoresed on 12.5 % SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL) by electroblotting overnight. Membranes were blocked with 10 % electrophoresis grade biotin-depleted non-fat dry milk (BioRad) in 1 X PBS (10 mM Tris pH 7.5, 100 mM NaCl, 0.1 % Tween-20), rinsed in PBS, probed with monoclonal mouse anti-human bcl-2 and p53 (Transduction Laboratories San Diego, CA) at 1:500 and 1:1000 dilution, respectively and washed X 3 in PBS. The secondary antibody was HRP-conjugated goat anti-mouse whole IgG used at 1:1000 (Transduction Laboratories). All antibodies were diluted in 1 % milk in TBS. Membranes were washed three times and detection was performed by enhanced chemiluminescence with an ECL reagent kit (using 0.06 ml/cm² of reagent) and Hybond autoradiography film (both Amersham). Biotinylated standards were used for molecular weight determination and were detected with 1:3000 streptavidin-horseradish peroxidase (Amersham). A Bio-Rad GS670 densitometer was used to determine the relative band intensity.

2.6. RNA preparation and analysis

RT-PCR was performed as previously described [38,40]. Briefly, total cellular RNA from peripheral blood T cells or T cell line (Jurkat) was obtained by guanidium isothiocyanate lysis and centrifugation through a CsCl₂ cushion. Three million cells were plated at 0.5 M/ml in 65 mm tissue culture dishes and stimulated with xenoestrogens. At the designated time of harvest cells were transferred to a 4 ml polypropylene tube, pelleted, washed twice in 1 X PBS and lysed in 3 ml guanidinium. Centrifugation was performed for 16 hr at 33K RPM. RNA was resuspended, precipitated and quantitated spectrophotometrically. Aliquots of RNA were run on an ethidium stained gel to ensure good quality. Reverse transcription (RT) of RNA was performed under standard conditions using equal μ g input RNA from each condition. The PCR reaction was performed under the following conditions in a 25ul reaction (95°C, 5'). Five ul of this reaction was used for PCR amplification in a 25ul reaction, representing amplification of cDNA generated from 0.25 μ g of total cellular RNA. The PCR reaction was performed under the following conditions in a 25ul reaction in a Perkin Elmer DNA Thermal Cycler: 10 pmoles each primer, 200 (M dNTP, 1.25 U *Taq*, 1 X *Taq* reaction buffer (all Promega) as previously described [40]. PCR primers were specific for human bcl-2,

upstream 5'GCATGAATTCCCTCTGGGAAGGATGGCGCACGC and

downstream 5'GATCGAATTCGTGGCTCAGATAGGCACCCAGGGTGATG and p53,

upstream 5'CTGAGGTTGGCTCTGACTGTACCACCATCC and

downstream 5'CTCATTCAGCTCTCGGAACATCTCGAAGCG.

PCR reactions were optimized for annealing temperature and were performed at various cycle numbers to ensure that the result obtained was from within linear range of the amplification curve. GAPDH was amplified from the same RT reaction as a control. Polaroid photos of ethidium stained gels were taken. The results are semiquantitative when performed in this fashion.

2.7. Statistical analysis

Data is presented as mean \pm standard error of the mean. Statistical analysis was performed by one-way ANOVA for multiple samples or by *Student-t* testing with matched pairing if appropriate.

3. Results

3.1 Xenoestrogen effects on Jurkat T cell proliferation

Study results show compound-specific effects on accumulation of viable T cell mass. 17- β -estradiol and TCDD were used as representative controls of endogenous and environmental estrogen compounds. In Figure 1, coumestrol and TCDD exhibited concentration dependent (10^{-4} to 10^{-7} M) suppression on Jurkat T cell proliferation, mimicking the cytotoxic effects of 17- β -estradiol [36-39]. In contrast, BPA and DDT (data not shown; see below) had essentially no effect on Jurkat T cell viability and cell mass accumulation over 72 hours exposure.

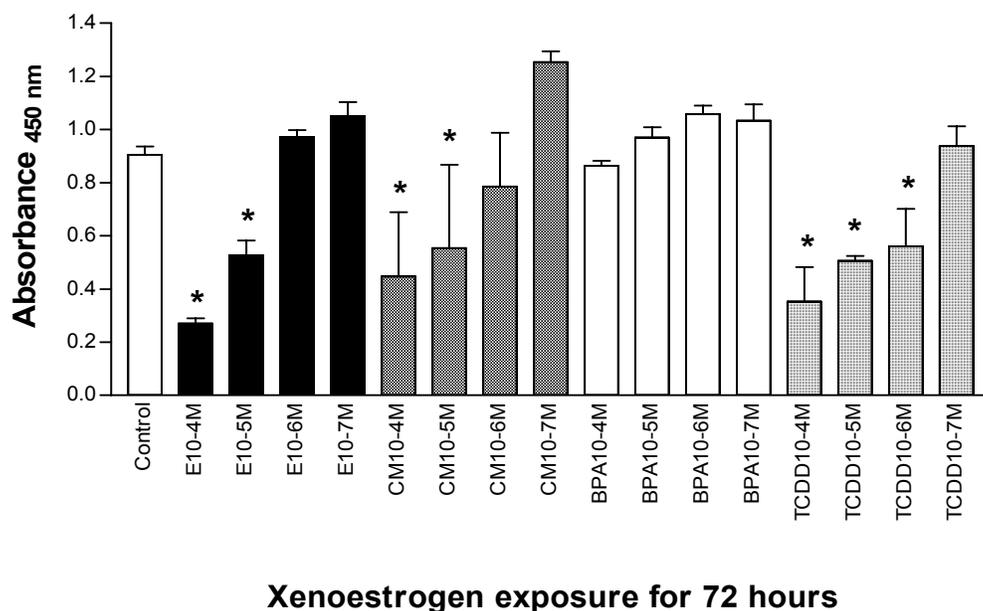


Figure 1. Jurkat T cell mass ($n = 3$) after 72 hours exposure to 17- β -estradiol (E), coumestrol (CM), bisphenol A (BPA), or TCDD at 10^{-4} to 10^{-7} M vs. control. Viable cell mass was assessed by MTT assay (see Methods). Change in total cell number was confirmed in cell cultures by enumeration (not shown). * $p < 0.05$ vs. control by ANOVA with Bonferroni correction for multiple comparisons. Xenoestrogens were dissolved in 1,4-dioxane or DMSO with final concentrations of solvent in control or treated cultures $< 0.1\%$. DDT results were similar to BPA (data not shown; see Figure 2).

In Figure 2, DDT, at equimolar concentrations to 17- β -estradiol (10^{-5} M), had no direct effect on Jurkat T cell viability (i.e. no difference between control and DDT). However, DDT antagonized the 17- β -estradiol suppressive effect on Jurkat T cell mass accumulation over 72 hours, demonstrating an anti-estrogenic effect. Similar estrogen antagonism was observed for BPA in Jurkat T cells (data not shown). Coumestrol did not exhibit a significant antagonism to T cell suppressive effects of 17- β -estradiol by this assay but was additive to estradiol's apoptotic effects (data not shown).

3.2. Xenoestrogen modulation of cell cycle phase distribution

As cell accumulation or growth is a homeostatic balance between proliferation and apoptosis [42,43], questions have been raised regarding the MTT assay as a measure of viable cell mass, especially for xenoestrogens [44]. Therefore, xenoestrogen effects on cell cycle phase Jurkat CD4+ T cells was also assessed by propidium iodide staining [38,39,41]. Representative cell cycle histograms for 17- β -estradiol, coumestrol and BPA are shown in Figure 3. 17- β -estradiol and coumestrol had significant cell cycle phase effect on actively growing Jurkat T cells, causing redistribution from G₀/G₁ to apoptosis ($p < 0.01$), whereas BPA and DDT had minimal effect, when analyzed by PI staining (cumulative results $n = 4$; Figure 4). For the purposes of presenting this data, results have been grouped into G₀/G₁, S/G₂M, and apoptotic fractions.

As significant effects occurred at high concentrations during short-term exposure, their biotoxicological relevance is obviously questioned [8,11]. However, in long term exposure, lower concentrations of coumestrol had substantial effects on Jurkat T cell cycle distribution as shown in Figure 5. Lower concentrations of coumestrol in long term exposure show increased cell accumulation in S phase and G₂M (G₂M shift from 10 % vs. 25 % in coumestrol exposed), similar to the effects of TCDD on in vivo activated T lymphocytes [45].

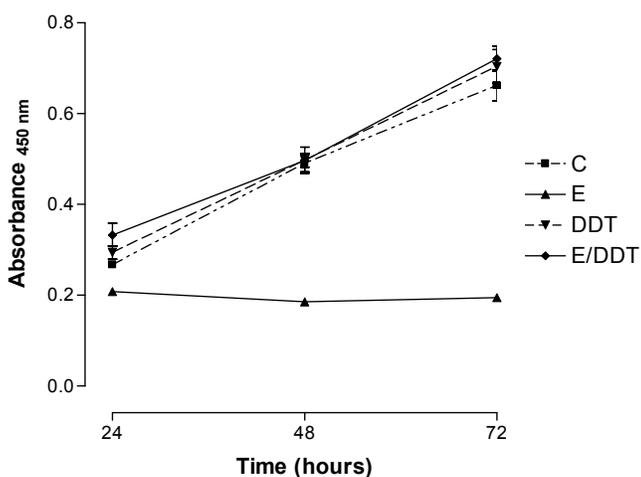


Figure 2. Jurkat T cell mass over 72 hours exposure to 10^{-5} M 17- β -estradiol (E), DDT (DDT), or combination 17- β -estradiol and DDT (E/DDT) vs. control (C). DDT alone had no effect on Jurkat T cell accumulation (no difference from control) but antagonized the suppressive effects of E at equimolar concentrations.

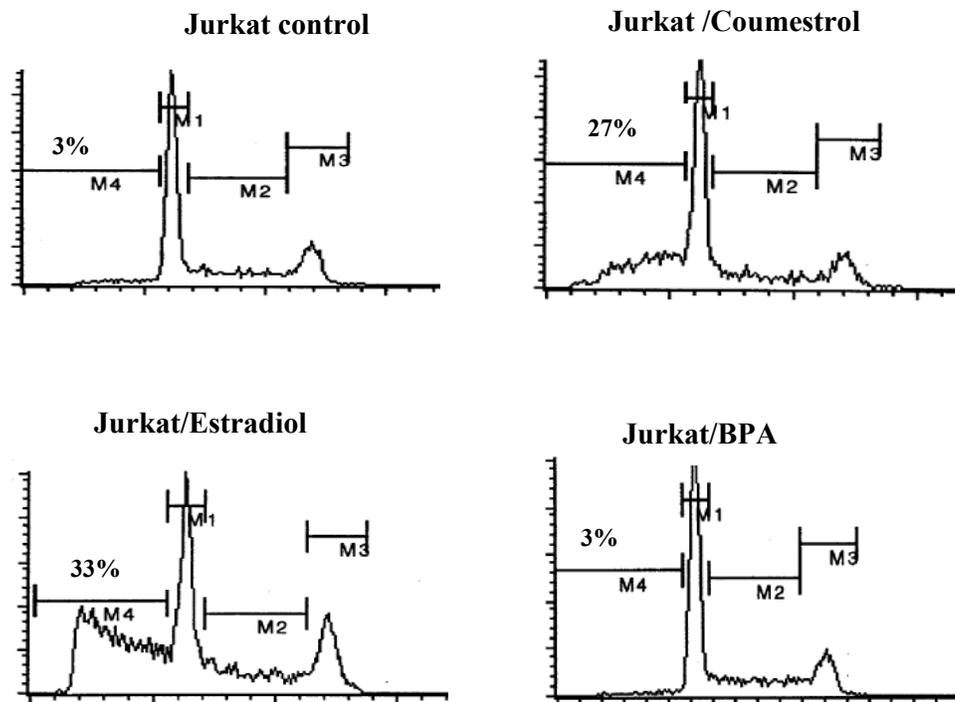


Figure 3. Representative cell cycle histograms of Jurkat T cells exposed to control or 10^{-5} M 17- β -estradiol (E), coumestrol (CM), and BPA for 72 hours as previously described [25,26]. Percent apoptotic cells (M4 range) is shown.

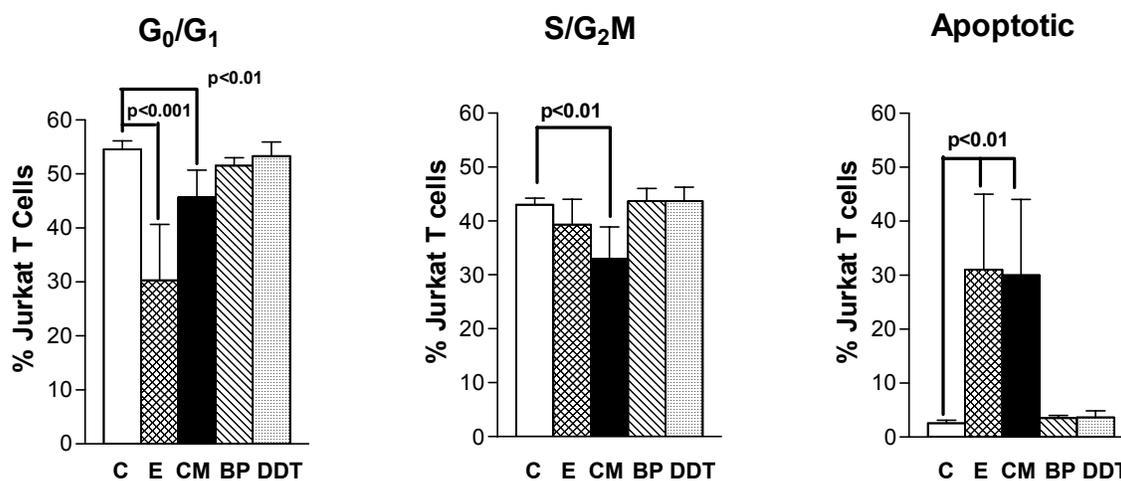


Figure 4. Cumulative effects of xenoestrogens on Jurkat T cell cycle phase distribution (n = 3) at 72 hours exposure of 10^{-5} M 17- β -estradiol (E), coumestrol (C), bisphenol A (BPA), and DDT vs. control (C). Percentage (mean \pm SEM) of cells in each phase and apoptotic fraction was determined by PI staining and analyzed by ANOVA for statistical significance.

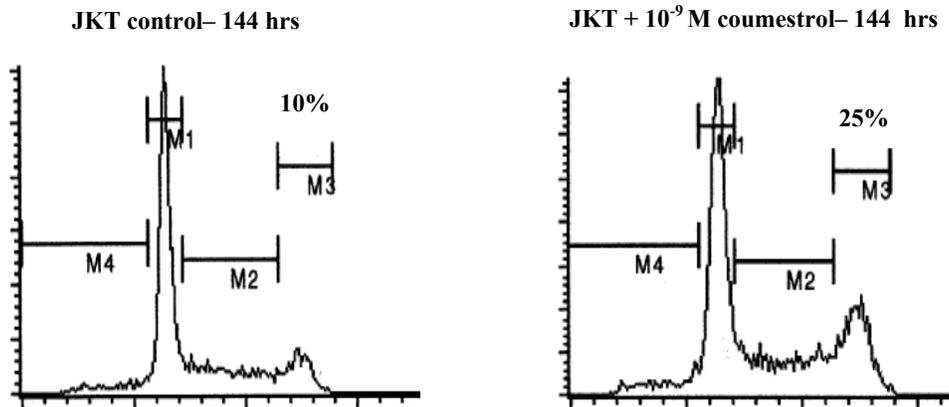


Figure 5. Representative PI cell cycle analysis of Jurkat T cells at 6 days culture in the presence of 10^{-9} M coumestrol compared to control culture. Coumestrol increased the percentage of cells in G_2M (M3 range) from 10 % to 25 %.

3.3. Xenoestrogen suppression of *bcl-2* and stimulation of *p53*

Bcl-2 and p53 cell regulatory proteins modulate cell cycle progression and apoptosis [42,43,46]. Given the observed effects of xenoestrogens on Jurkat T lymphocytes, examination of bcl-2 and p53 protein levels was performed. As shown in representative Western blots (Figure 6; n=3), xenoestrogens suppressed bcl-2 and increased p53 protein levels to variable degrees in Jurkat cells. Suppression of bcl-2 or augmentation of p53 for the xenoestrogens was concentration dependent from 10^{-8} to 10^{-4} M (data not shown). These preliminary results are consistent with recent reports of potential modulation of bcl-2 and p53 proteins by xenoestrogens [47-50], supporting the concept that xenoestrogens may modulate T lymphocyte biology through associated changes in bcl-2 and p53.

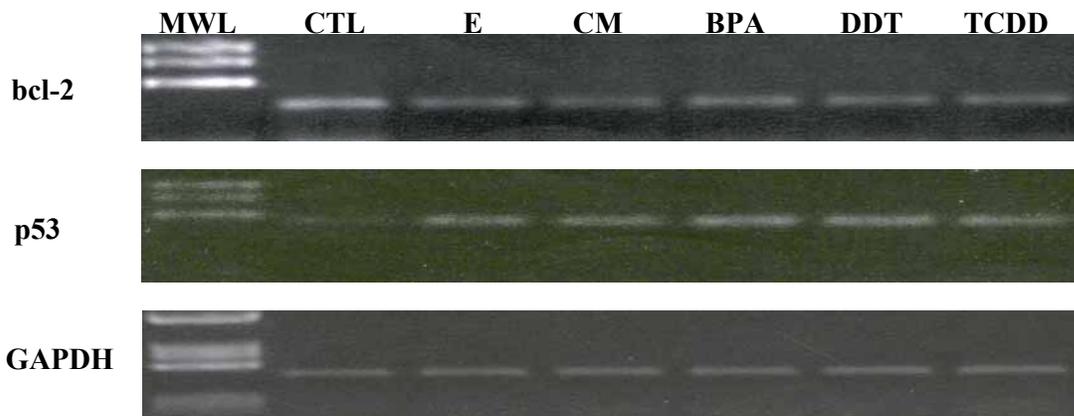


Figure 6. Western blot analysis of bcl-2 and p53 protein in activated Jurkat T cells exposed for 24 hrs to 17- β -estradiol (E), coumestrol (CM), bisphenol A (BPA), DDT or TCDD at 10^{-6} M vs. control (CTL) performed as previously described [38,39]. Values are fold change arbitrary densitometry units relative to CTL (CTL= 1.0).

Xenoestrogen modulation of bcl-2 and p53 protein levels may occur at the level of mRNA transcription or degradation. Preliminary examination of this hypothesis was achieved by semiquantitative PCR as described [38,39]. As shown by the representative experiment (Figure 7; n = 3), xenoestrogens suppressed bcl-2 mRNA transcript levels with stable GAPDH transcripts. In contrast, p53 mRNA transcripts were increased by xenoestrogens compared to non-exposed Jurkat T cells (Figure 7). These preliminary results imply that xenoestrogens have gene specific effects on cell cycle regulatory proteins that may determine cell cycle phase distribution or induction of apoptosis.

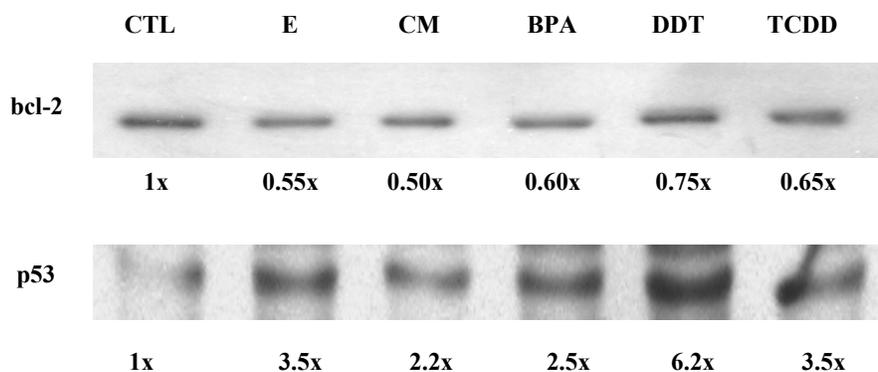


Figure 7. RT-PCR of bcl-2 and p53 total Jurkat T cell RNA after 24 hours exposure to 10^{-6} M 17- β -estradiol (E), coumestrol (CM), bisphenol A (BPA), DDT or TCDD. RT-PCR and gel electrophoresis as described in Methods.

3.4. Xenoestrogen effects on apoptosis

Lymphocyte cell mass accumulation, cell cycle phase distribution, and apoptosis were variably modulated by coumestrol, BPA, DDT, and TCDD. Apoptotic effects of xenoestrogens were further assessed by DNA fragmentation (laddering) as previously described. As shown in a representative experiment (Figure 8, n = 3), coumestrol, BPA, DDT and TCDD (10^{-4} M) induced demonstrable DNA fragmentation, suggesting apoptosis induction in Jurkat T lymphocytes. Induction of DNA fragmentation by BPA and DDT was unexpected as MTT cell mass assessment and PI cell cycle histograms had not detected significant cell death upon exposure to these xenoestrogens. Complex processes, such as increased proliferation or cell cycle redistribution masking this xenoestrogen effect may be responsible for this disparity; alternatively, simpler explanations such as sensitivity and specificity of assay methods may explain the differences between results.

3.5. Xenoestrogen effects on peripheral blood mononuclear cells

In extension of these results, xenoestrogen effects on cell cycle distribution in purified peripheral blood lymphocytes (PBLs) were examined. Preliminary characterization of xenoestrogen effects on PBLs demonstrated significant changes in cell cycle phase distribution, upon exposure to 10^{-6} M 17- β -

MWL CTL CM BPA DDT TCDD

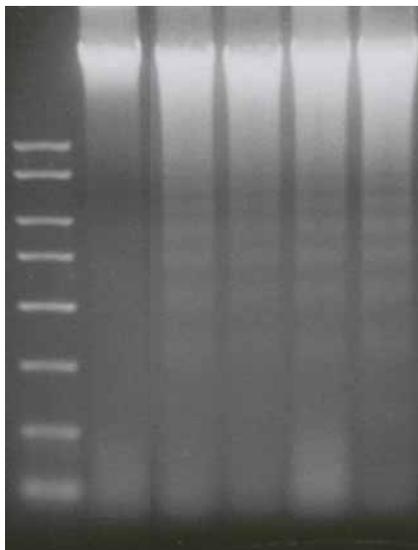


Figure 8. DNA Fragmentation and apoptosis in xenoestrogen exposed Jurkat T lymphocytes. After 72 hours incubation, there is no significant apoptosis in control Jurkat cells (CTL) but DNA nuclear fragmentation in Jurkat cells exposed to 10^{-5} M coumestrol (CM), BPA (BPA), DDT (DDT) and TCDD (TCDD).

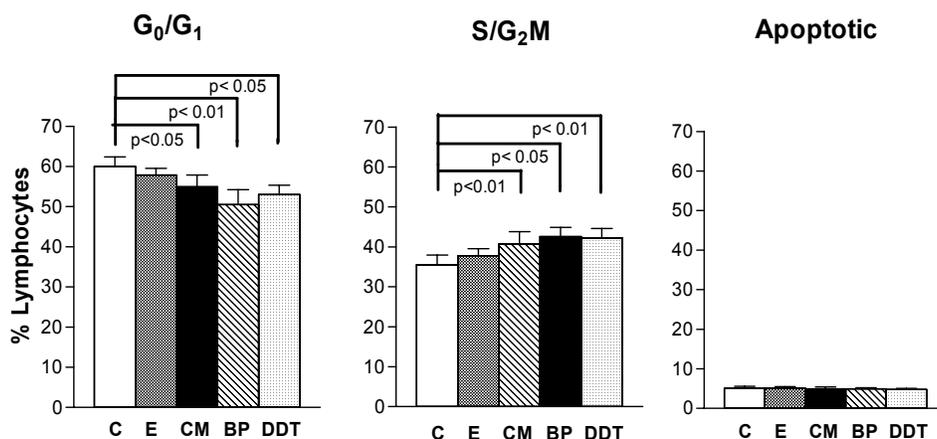


Figure 9. Cell cycle analysis of α CD3/PMA (10^6 cells/ml) activated PBLs ($n = 10$ samples) exposed for 72 hours in culture to 10^{-6} M 17- β -estradiol (E), coumestrol (CM), bisphenol A (BP), or DDT (DDT) compared to control (C). Percentage of lymphocytes in each phase and the apoptotic fraction was determined by PI staining and statistical differences in values (mean \pm SEM) were analyzed by ANOVA with correction for multiple comparisons.

estradiol (E), coumestrol (CM), bisphenol A (BP) or DDT for 72 hours (cell cycle data is shown in Figure 9). Significant xenoestrogen-induced exodus of PBLs from G₀/G₁ and accumulation in S/G₂M was observed, consistent with previously documented effects of TCDD exposure on murine lymphocytes in vivo [45] and the low concentration, long term effects of coumestrol shown in Figure 4.

4. Discussion

Endogenous estrogens are immunoregulatory [1-5], with their actions generally characterized as suppressive to cell mediated immunity [36,37]. Xenoestrogens are environmental compounds with an estrogenic agonist/antagonistic activity known to disrupt the hypothalamic/pituitary/gonadal axis [6-12]. A classic xenoestrogen, TCDD suppresses lymphoproliferation and induces apoptosis in immature lymphocytes [29-31] and was used with the endogenous estrogen 17- β -estradiol as standards for xenoestrogenic effects in this study. Direct or indirect immune effects of other xenoestrogens such as coumestrol, bisphenol A, and DDT have been less well characterized and yet may have significant impact for cell mediated immunity, reproductive immunology, or autoimmune disease [6-13]. Although the concentrations in this investigation were higher than those found in the environment, long-term bioaccumulative actions may be anticipated to affect lymphocyte biology. With receptor binding affinities up to 10,000 fold weaker than endogenous estrogens, the environmental impact of xenoestrogens is the subject of scientific and societal debate [11-15,51]. These preliminary studies of representative xenoestrogens have been designed to detect and dissect potential immunoendocrine effects on Jurkat T lymphocytes. As a test model, Jurkat T cells are transformed CD4+ lymphocytes expressing the T cell receptor, CD3, and a number of mature T cell markers. Jurkat T cells produce IL-2 and exhibit a cell cycle profile in response to activation in a manner resembling peripheral blood T cells [52-55].

Coumestrol, at concentrations used in this study, suppressed Jurkat T cell lymphoproliferation and was associated with suppression of resting (G_0/G_1) phase, similar to actions seen for the endogenous estrogen, 17- β -estradiol [38,39]. Coumestrol exposure produced apoptosis of Jurkat lymphocytes in association with suppressed bcl-2 and increased p53 protein levels. To our knowledge, the effects of coumestrol on T lymphocytes and bcl-2/p53 have not been previously characterized.

BPA induced apoptosis as detected by DNA fragmentation, but did not have a significant effect on cell mass accumulation or cell cycle phase distribution, suggesting a balanced effect on lymphoid cell death and survival. BPA did suppress bcl-2 and induce p53 protein and mRNA levels. BPA effects on lymphoid cells have also not been well characterized, so clearly preliminary results in this study require verification.

DDT antagonized 17- β -estradiol actions on lymphocytes, with an induction of DNA fragmentation and suppression of bcl-2 observed; however, minimal effects on cell cycle phase distribution occurred. As with BPA, this synthetic xenoestrogen likely has a multitude of effects whose cellular effects, *in toto*, may reflect the sum result of effects on multiple cell regulatory proteins. Given its effects on DNA fragmentation, bcl-2, and PBL cell cycle, DDT exposure has similar effects to that of 17- β -estradiol on lymphohematopoietic cells [38,39]. The observed DDT antagonism of estrogen-induced apoptosis in lymphocytes in the current study is consistent with a previous report of its anti-apoptotic effect in a breast cancer cell line [50]. While pro-apoptotic effects have been documented with the related xenoestrogen, endosulfan, this compound was associated with increased bcl-2 protein

expression, suggesting that its mechanism of action was bcl-2 independent [47]. Endogenous 17- β -estradiol not only suppresses bcl-2 but also inhibits microtubule function [38,39]. It is therefore likely, and even expected, that xenoestrogenic compounds are only superficially related and will not all have similar effects or mechanisms of action. This paradigm is similar to the compound specific effects of estrogens and estrogen receptor blockers (e.g. tamoxifen), which have pro- or anti-estrogenic effects that are tissue-type specific [56,57].

TCDD was examined adjunctively in this study as a standard xenoestrogen. In this study, its suppression of lymphocyte cell mass accumulation and induction of DNA fragmentation indicative of apoptosis were confirmed and its modulation of bcl-2 and p53 protein levels documented. TCDD has a wide variety of immunomodulatory effects at both the cellular and molecular levels. It has been shown to induce apoptosis in immature lymphoid cells [29,30], that may be bcl-2 independent [58], and to induce expression of p53 protein [28], which may or may not be associated with apoptosis. The current investigation expands the number of xenoestrogen compounds demonstrating effects on lymphocyte biology similar to effects of TCDD.

A uniform effect of these ecohormones on cell regulatory proteins is befitting their characterization as xenoestrogens; however, this is an oversimplification. Resembling the actions of endogenous estrogens, these environmental estrogens likely have variable and specific target-organ effects. The phenomenon described herein must be viewed with respect to lymphocytes, as preliminary studies of xenoestrogen-exposed monocytes did not produce similar results (data not shown). The concentrations of these xenoestrogens were high and used purposefully to detect an effect that may or may not be applicable to typical environmental situations. Nevertheless, lower concentration longer-term exposure and observations of effects on PBLs suggest that these xenoestrogens could have a biological impact individually or in combination. Effects in the current study were confined to direct *in vitro* lymphocyte modulation; clearly, these effects would be more complicated *in vivo* when disrupting other immunomodulatory hormones such as testosterone or prolactin [59,60]. Speculation linking these xenoestrogens to epidemiological data on infectious, neoplastic, or immune disease is hampered by the relatively limited range of understanding of the *in vitro* and *in vivo* T lymphocyte biological activities of these compounds. The demonstration of direct xenoestrogen effects on T lymphocytes facilitates the development of incisive studies of mechanisms of action of these immunodulatory xenobiotics.

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