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DDT Induces Apoptosis in Rat Thymocytes

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In general, exposure of cells to high concentrations of toxic agents results in non specific mode of cell death, termed necrosis. In contrast, apoptosis, a physiological form of cell death, often triggered by a variety of external stimuli, proceeds in orderly and controlled morphological and biochemical steps (Wyllie et al, 1980: Cohen et al, 1992). The sequence of these events leading to cell death is well known and is characterized, in particular, by chromatin condensation and internucleosomal DNA cleavage while nectrotic cells are characterized by the swelling of cytoplasm but not nuclear condensation and DNA fragmentation (Cervinka and Puza, 1995).

Apoptosis can be induced by a wide variety of stimuli such as glucocorticoids, radiation and xenobiotics (Weaver et al, 1996). DDT (1,1 bis (p-chlorophenyl) 2,2,2 trichloroethane) has been the most widely used organochlorine pesticide in many countries throughout the world. The high lipophilicity and stability of this compound contribute to its persistence as an environmental pollutant resulting in a significant contamination of human food although it has been banned for a long time (Ferrer and Cabral, 1995). Organochlorine insecticides have undesirable effects on reproduction (Multigner et al, 1996) and act as immunotoxicant increasing the risk of carcinogenesis (Hoffmann 1996). In addition chlorinated hydrocarbons such as 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and endrin, have been shown to induce lipid peroxidation and enhance nuclear DNA damage (Wahba et al, 1989; Bagchi et al, 1992).

The objective of this study is to examine the cytotoxic effect of the DDT on the well-characterized cell system of rat thymocytes and to evaluate whether apoptosis is involved in this cytotoxicity.

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MATERIALS AND METHODS

DDT (98% pp') and all other reagents are obtained from Sigma Chemical Co (St Louis, USA). Agarose was obtained from GibcoBRL (Paisley, Scotland). Male Wistar Rats (100-150 g) bred in our laboratory were allowed food and water *ad libitum*. They were sacrified by decapitation at the age of 4-6 weeks.

To prepare thymocytes, thymuses were removed and placed in ice cold phosphate buffered saline (PBS: 140 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) dissected and dissociated using a Potter homogeniser. The cell suspension was then filtered through Screen filter 70 mesh (Sigma) to remove debris. After washing twice with PBS, the cells were diluted to a final density of 10^7 cells/ml supplemented with 10% heat-inactivated fetal calf serum and containing penicillin (100 units/ml) and streptomycin (100 µg/ml). Viability, determined by trypan-blue exclusion, was greater than 95%. Isolated thymocytes were incubated for 4 and 6 hours either alone (controls), with dexamethasone (Dexa) (10^{-5} M), or with DDT (2.10^{-5} M) in 95% 02, 5% CO_2 atmosphere in a final volume of 1 ml/ plate. Where indicated $ZnSO_4$ was added at a concentration of 1 mM. The pesticide was dissolved in 50% ethanol before added to the medium. The final concentration of ethanol was lower than 0.01% and had no detectable effect on cells.

DNA extraction and electrophoresis have been performed according to the method described by Genaro et al (1995). Briefly, at the end of the incubation period, thymocytes were centrifuged at 200 x g for 10 min at 4°C and the pellet was washed twice with ice-cold PBS. The pellet was resuspended in lysis buffer (5 mM tris-HCl, 20 mM EDTA, 0.5 % triton X-100, pH 8) for 15 min at 4°C. Nuclei were removed by centrifugation at 500 x g for 10 min and the resulting supernatant was centrifuged at 30,000 g for 15 min. The fragmented DNA present in the soluble fraction was precipitated with 70% ethanol plus 0.1 vol. of 3 M ammonium acetate and aliquots were treated for 1 h at 55°C with 0.3 mg/ml of proteinase K then with RNase for 30 min at 25°C. After two extractions with phenol/ chloroform, the DNA was resuspended. DNA concentrations were determined by spectrophotometric absorption at 260 nm. 3 µg of DNA/ sample was loaded into 1.5% agarose gel in 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA (pH 8.0). DNA was visualized using UV illumination (260 nm) after ethidium bromide staining.

Data were analyzed using Stat View 512$^+$ software (Abacus Concept,

Inc). Means were given with standard error (SE) and were subjected to the unpaired Student's *t*-test.

RESULTS AND DISCUSSION

The effect of various concentrations of DDT on thymocyte viability was examined after 4 and 6h of incubation (Table 1). By 4 h of incubation and except for 2.10^{-4}M concentration of pesticide, cell viability was not significantly altered. After 6h of incubation with 2.10^{-5} and 2.10^{-4}M DDT cell viability decreased to 70 and 57% respectively in comparison to unexposed control cells. Whereas viability remained more than 90% of control value with 10^{-9}M and 10^{-7}M of pesticide.

Table 1. The effect of DDT at various concentrations on the thymocytes viability at 4 and 6h of incubation.

DDT Concentration	4h	6h
Control	98.0 ± 0.4	96.7 ± 0.2
10^{-9}M	97.4 ± 0.2	96.3 ± 0.1
10^{-7}M	96.2 ± 0.2	93.9 ± 1.0
2.10^{-5}M	83.9 ± 1.0	70.2 ± 2.4*
2.10^{-4}M	78.4 ± 0.6*	57.6 ± 5.4*

Results shown are the mean values ± SE from two separate experiments done in triplicate.
* $p < 0.05$ compared to control.

Since glucocorticoids have a well known apoptotic effect on thymocytes (Brown et al, 1993; Schwartzman and Cidlowski, 1994), we have compared the effect of DDT to that of the synthetic glucocorticoid Dexa. Figure 1 illustrates the time-course of viability of thymocytes incubated with 10^{-5}M Dexa or 2.10^{-5}M DDT. The treatment of cells with the glucocorticoid or the pesticide results in a similar time-dependent decrease in cell viability reaching less than 70% of control value by 6 h of incubation. While spontaneous cell lethality in control cultures was less than 3% during the same period. On a molecular basis, DDT seems to be less potent than Dexa to induce thymocytes death. This suggestion is supported by *in vivo*

experiments demonstrating that DDT treatment for 10 days caused a dose-dependent thymic atrophy reaching 31% at 100 mg/kg body weight (figure 3). While, in a previous study, we reported that a single injection of Dexa (1,5 mg/kg) resulted 4 days after in a marked thymic weight loss of about 70-80% (Ben Rhouma and Sakly, 1994). Indeed, in terms of the magnitude of their thymotoxicity, DDT also showed a lower *in vivo* toxic potency than Dexa.

Our results on the *in vitro* cytotoxic effect of Dexa agree with the finding of Provinciali et al (1995) who found a comparable percent viability of cultured mouse thymocytes 8h after exposure to Dexa but with steroid concentrations two orders of magnitude lower than that used in the present work. Whereas, other studies show a greater loss of cell viability in presence of the glucocorticoid. But in contrast to our results, these studies reported a higher spontaneous cell death (Zucker et al, 1994). Addition of 1 mM $ZnSO_4$ prevented both Dexa- and DDT-induced cell death. Cell viability remained more than 80% of control after 6h of incubation. The supplementation with zinc did not significantly decrease the number of viable cells in control cultures.

These findings agree with previous studies showing an inhibition by zinc of thymocytes apoptosis induced by either Dexa (Cohen and Duke, 1984), irradiation (Sellins and Cohen, 1987) or heat-shock (Migliorati et al, 1992). This protective effect of zinc on apoptosis has been assumed to be due to the ability of the metal ion to inhibit a Ca^{2+}/Mg^{2+}- dependent endonuclease which is responsible for the DNA fragmentation in apoptotic cells (Lohmann and Beyrsmann 1995). Zinc is also an antioxidant. It potentiates the effect of diethyl-dithiocarbamate, an antioxidant thiol, to inhibit radiation-induced apoptosis in thymocytes (Mathieu et al, 1996).

Agarose gel electrophoresis of cellular DNA is the usual method for demonstrating apoptosis. The cleavage of DNA into nucleosomal size fragments of 180-200 base pairs or multiples thereof result in a characteristic DNA ladder pattern.(Wyllie, 1980; Squier et al, 1995). As shown in figure 2, DNA fragmentation is negligible in thymocytes immediately after isolation and for control cells after 6h of incubation in free medium, whereas thymocytes exposure to DDT resulted in a DNA ladder, typical of apoptosis, similar to that observed in Dexa-treated cells. Furthermore, as for Dexa, DNA fragmentation induced by DDT is inhibited by zinc suggesting a common pathway for these two apoptotic factors.

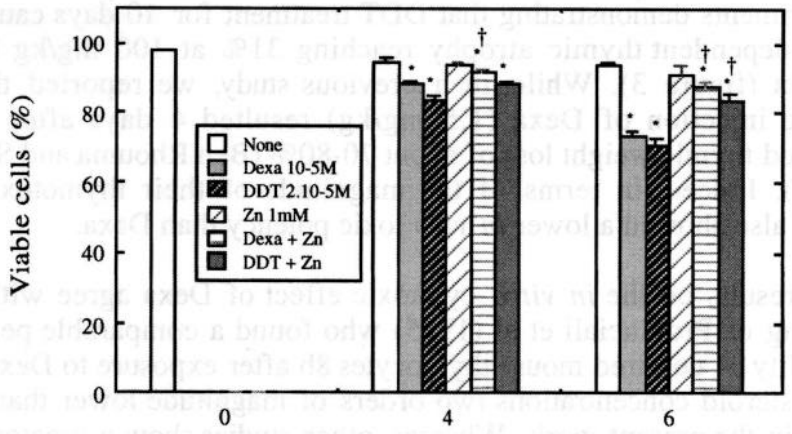

Figure 1. Percentage of viable thymocytes after 0, 4 and 6h of incubation in free medium or containing Dexa (10^{-5}M) or DDT (2.10^{-5}M) with or without 1 mM $ZnSO_4$(1 mM).
* $p < 0.05$ compared to control, † $p < 0.05$ compared to Dexa or DDT.

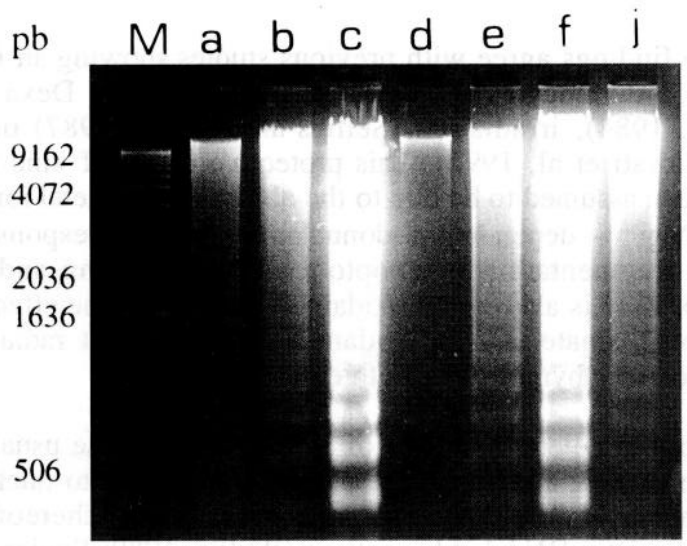

Figure 2. Agarose gel analysis of DNA samples obtained from thymocytes not incubated (lane a) and incubated 6h in control medium (lane b), medium containing 10^{-5}M Dexa (lane c), 1 mM Zinc (lane d), 10^{-5}M Dexa plus 1 mM Zinc (lane e), 2.10^{-5}M DDT (lane f), 2.10^{-5}M DDT plus 1 mM $ZnSO_4$(lane j). 1 kb molecular weight DNA marker (lane M).

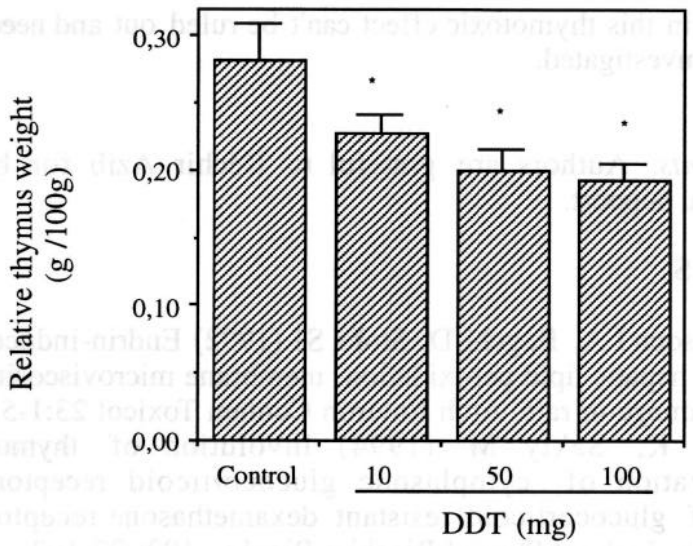

Figure 3. Effect of *in vivo* DDT treatment on thymus weight.
DDT (10, 50 or 100 mg/kg body weight) is administrated i.p in corn oil, control animals received the vehicle. Each value is the mean ± SE of 5 determinations.
* $p < 0.05$ compared to control.

In addition, zinc completely inhibits the cleavage of DNA into oligonucleosomal fragments but does not prevent the cleavage of DNA into high molecular weight fragments in thymocytes exposed to either DDT or Dexa. This observation supports the suggestion that key enzyme (s), other than Ca^{2+}/Mg^{2+}dependent endonuclease, are involved at the earliest stages of induction of apoptosis (Brown et al,1993)

Thus, our data suggest that DDT-induced cell death in thymocytes is mediated by apoptosis. By this apoptotic effect, DDT could have a profound immunotoxic action similar to that caused by corticosteroid hormones (Schwartzman and Cidlowski, 1994) and dioxin (Lundberg, 1991). However the mechanism by which it exerts this toxic effect remains unknown. Several studies indicate that DNA damage induced by xenobiotics is a consequence of oxidative cellular alteration which subsequently induces endonuclease activity resulting in DNA fragmentation (Wahba et al, 1989; Marczynski et al, 1997).

Although the DDT sample is 98% pure, the possibility that the 2% contamination with contains the isomer op' and different metabolits

play some role in this thymotoxic effect can't be ruled out and needs certainly to be investigated.

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