Chapter 2: Evidence for the Induction of Apoptosis in Thymocytes by 2, 3, 7,8-tetrachlorodibenzo-p-dioxin (TCDD) *in vivo*.

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Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is well known for its immunotoxic effects particularly on the thymus. The exact mechanism by which TCDD induces thymic atrophy is not clear. In the current study, we investigated whether TCDD triggers apoptosis in thymocytes, when administered *in vivo*, by using TdT- mediated FITC-dUTP nick end labeling (TUNEL) method and analyzing the cells flow cytometrically. Significant apoptosis was detected at 8-12 hours after the TCDD injection but not at 24 hours or beyond, up to 120 hours of study. Furthermore, the induction of apoptosis was confirmed using JAM test in which thymocytes from TCDD-treated mice, labeled with ^{3}H-thymidine exhibited increased DNA fragmentation when compared to the controls. Similar to TCDD-treatment, administration of dexamethasone (5 or 100 mg/kg) into C57BL/6 mice triggered significant apoptosis that was detected only at 12 hours after administration of the drug but not thereafter. When thymocytes from TCDD or dexamethasone-treated mice were cultured *in vitro* for 24 hours, they exhibited marked increase in apoptosis when compared to the vehicle-treated controls. However, TCDD, when added to *in vitro* cultures of thymocytes, failed to trigger apoptosis. Together, our studies demonstrate that TCDD can induce apoptosis in thymocytes *in vivo*. This can be detected only at an early stage following TCDD administration, possibly because of rapid clearance of apoptotic cells by the phagocytic cells *in vivo*.

Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is well known for its immunotoxic effects particularly on the thymus. Despite extensive research, the exact mechanism by which TCDD induces thymic atrophy remains unclear. There are mainly three hypotheses proposed to account for the susceptibility of the thymocytes to TCDD-induced toxicity. First, TCDD may cause thymic atrophy by destroying committed prethymocytes found in the bone marrow and fetal liver (Fine *et al.*, 1989, 1990). Secondly, TCDD may inhibit the intrathymic T cell development particularly at the double-positive (DP, $CD4^{+}CD8^{+}$) stage of T cell differentiation. This may result from either a direct effect on DP thymocytes or an indirect effect by arresting the differentiation of T cells that give rise to the DP cells (Blaylock *et al.*, 1992; Holladay *et al.*, 1991; Kerkvliet and Brauner, 1990; Lundberg, *et al.*, 1990). Alternatively, Greenlee *et al.* (1985) demonstrated that the thymic epithelial cells may be the primary targets of TCDD-induced immunotoxicity. Lastly, McConkey *et al.* (1988), suggested that TCDD may kill immature thymocytes by initiating apoptosis.

Apoptosis is a common form of eukaryotic cell death resulting from the DNA fragmentation into oligonucleosomal length fragments. Recently, several chemicals were shown to induce apoptosis in thymocytes (Comment *et al.*, 1992). However, these studies failed to demonstrate loss of cell viability following addition of TCDD to *in vitro* thymocyte cultures and therefore were unable to confirm the previous studies by McConkey *et al.*, (1988).

Although TCDD may fail to induce apoptosis *in vitro*, whether it does so *in vivo*, remains a possibility because the demonstration of apoptosis *in vivo* is difficult due to the rapid clearing of apoptotic cells (Savill *et al.*, 1993; Surh and Sprent, 1994). This may be one of the reasons why in a recent study, Silverstone *et al*., (1994) failed to

detect TCDD-induced apoptosis *in vivo* when studied at 48 hours or later, following TCDD administration. To overcome this problem, recently we used C57BL/6 lpr/lpr mice that are deficient in the expression of Fas (CD95), a molecule considered to play an important role in the induction of apoptosis in cells (Itoh *et al.*, 1991). Using such mice, we were able to demonstrate that TCDD was less toxic to the thymocytes in Fas-deficient mice when compared to the Fas^{+} wild type mice (Rhile *et al*., 1996). In the current study, we provide direct evidence suggesting that TCDD does trigger apoptosis of thymocytes *in vivo*.

Experimental Procedures

Mice:

Adult, female C57BL/6 mice were purchased from National Institutes of Health, Bethesda, MD. The mice were housed in polyethylene cages containing wood shavings in laminar flow units (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) and given rodent chow and tap water *ad libitum.* Mice were housed in rooms maintained at a temperature of 74 ± 2^0 F and on a twelve hour light/dark cycle.

TCDD exposure:

2,3,7,8-Tetrachlorodibenzo-p-dioxin was generously provided by Dr. K. Chae of NIEHS, Research Triangle Park, NC and stored at -20^0 C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone (Rhile *et al.*, 1996). Mice were administered various doses of TCDD or the vehicle, by a single intraperitoneal injection.

Dexamethasone exposure:

Dexamethasone was purchased from Sigma Chemical, MI and was dissolved in DMSO and made up to the required doses of 5 or 100 mg/kg body weight and administered through a single intraperitoneal injection. The control mice received DMSO alone.

Cell preparation:

Mice were euthanized after TCDD, dexamethasone or vehicle treatment and the thymus was surgically removed. The thymus was placed in RPMI-1640 medium (Gibco Lab., Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM HEPES, 1 mM glutamine, 40 μg/ml of gentamicin sulfate and 50 μM 2-mercaptoethanol, referred to as complete tissue culture medium. Single cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co.,

Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation and resuspended in 0.83% ammonium chloride to lyse the erythrocytes. Cells were further washed twice in medium.

Total cellularity:

Thymocytes were prepared as described above and resuspended in medium. Twenty five microliters of the single cell suspension was added to 100 or 500 μl of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under an inverted phase contrast microscope.

Detection of apoptosis using TUNEL method:

In the current study, we used a kit for the detection and quantification of apoptosis at single cell level, which is based on labeling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly referred to as TdT-mediated nick end labeling or TUNEL technique (Boehringer Mannheim, Indianapolis, IN). The cells (5×10^6) were washed twice with medium containing phosphate buffered saline (PBS) and fixed with 4% p-formaldehyde for 30 minutes at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP for an hour in the incubator. Fluorescence of the cells was measured by flow cytometry as described (McKallip *et al.*, 1995). The analysis was performed with a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

Detection of apoptosis using gel electrophoresis:

This procedure was carried out as described in detail elsewhere (Xu *et al*., 1993). Fifty million thymocytes were used to isolate DNA. Cells were lysed in a buffer containing 10 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS and 0.2 mg/ml proteinase K and then centrifugated to separate the chromatin and the DNA fragments. Samples were extracted and purified sequentially with phenol, phenol:chloroform (1:1), twice, and

chloroform followed by precipitation in 100% ethanol at -20^0 C overnight. The DNA was then quantitated by using a spectrophotometer (260 nm). A fixed amount of the DNA (8 μg/lane) was then run on 2% agarose gels. The gel was stained with ethidium bromide and observed under UV light.

Quantitation of DNA fragmentation by the JAM test:

JAM test was used to detect apoptosis in the thymocytes after suitable modifications, as described by others (Matzinger, 1991; Zhu and Anasetti, 1995). Thymocytes (25×10^6 cells) were labeled with [^{3}H] thymidine at a final concentration of 5 μCi/ml in complete medium for 4 hours. The excess [^{3}H] thymidine was washed off and the cells were plated into 96-well flat bottom tissue culture plates. At desired times, the cells were harvested onto glass fiber filters and counted in a liquid scintillation counter (Rhile *et al*., 1996). In this assay, the fragmented DNA from apoptotic cells gets washed away through the filters. Thus, the radioactivity present on the disc, corresponds to the intact DNA. The percent DNA fragmentation was calculated as:

% DNA fragmentation = (C-E)/C x 100; where C = cpm in retained DNA in the control sample and E = cpm in retained DNA of experimental sample.

Detection of apoptosis of thymocytes *in vivo* and *in vitro*:

Groups of 4-5 mice were injected with TCDD or dexamethasone and at various time intervals, the thymocytes from these mice were harvested, pooled and studied for the presence of apoptotic cells using flow cytometry. In these experiments, thymocytes from vehicle treated mice were used as a control. In some experiments, thymocytes were cultured for 24 hours in complete tissue culture medium and analyzed for the apoptotic cells. Also, to investigate whether addition of TCDD or dexamethasone to *in vitro* thymocyte cultures would trigger apoptosis, various concentrations of TCDD dissolved in DMSO or water soluble dexamethasone (Sigma Chemical, MI) was added to 50×10^6 thymocytes cultured in complete tissue culture medium in small tissue

culture flasks. At various time intervals thereafter, the cells were harvested, washed in PBS twice and analyzed for apoptotic cells using flow cytometry.

Statistical Analysis:

Each experiment was repeated at least twice, often more than 4-5 times to confirm the apoptosis. The total cellularity of thymocytes at various time intervals following chemical treatment was compared with the controls, using ANOVA and $p < 0.05$ was considered to be statistically significant.

Results

***Thymus cellularity in mice treated with dexamethasone or TCDD*:**

In the first set of experiments, we investigated the kinetics of the decrease in thymic cellularity induced by TCDD when injected into C57BL/6 mice. Groups of 5-6 mice were either injected with the vehicle or with 50 μg/kg body weight of TCDD intraperitoneally and after 12, 24, 48, 72 and 120 hours, the thymus was removed from these mice and the viable cell count was determined by the uptake of trypan blue dye. The total number of cells per thymus was calculated and is depicted in Table 1. In addition to TCDD, we also compared the effect of dexamethasone which has been previously shown to trigger apoptosis in thymocytes (Silverstone *et al.*, 1994). In all dexamethasone studies, we used two concentrations of dexamethasone, a low dose (5 mg/kg body weight) and a high dose (100 mg/kg body weight). In these studies, mice administered with DMSO were used as a control. The data depicted in Table 1 suggested that TCDD induced a delayed thymic atrophy, inasmuch as, decreased thymic cellularity was seen only 120 hours after TCDD administration, consistent with other studies (Fine *et al.*, 1990; Poland and Glover, 1980).

In contrast, when high dose of dexamethasone was administered into C57BL/6 mice, dramatic decrease in thymic cellularity was observed at 12 hours and the thymic atrophy continued to persist even at 72 hours after the initial administration (Table 1). However, with a lower dose, (5 mg/kg body weight) the induction of thymic atrophy was much delayed and was first demonstrable at 48 hours after the dexamethasone administration and the thymic cellularity was restored by 120 hours (Table 1). These data demonstrated that high dose of dexamethasone caused an early and persistent decrease in thymic cellularity, whereas, low doses of dexamethasone caused a less marked, delayed and transient decrease in thymic cellularity. Inasmuch as, lower doses of dexamethasone induce delayed thymic atrophy, somewhat similar to that triggered by TCDD and because dexamethasone is well known for its property to induce apoptosis, we used this drug in all subsequent experiments for comparison with TCDD.

Table 1

Thymic cellularity in mice treated with TCDD or dexamethasone.

Total thymocytes x 10^6/mouse[a]

Hours	Oil	TCDD (50 μg/kg)	DMSO	Dexamethasone	
				5 mg/kg	100 mg/kg
12	69.48±14.07	64.16±9.85	62.42±6.01	65.5±4.65	7.8±.737*
24		79.37±13.19		57.9±10.16	2.46±.136*
48		46.33±10.96		30.5±4.75*	1.38±.313*
72		37.18±3.8		28.9±10.05*	1.55±.176*
120		30.18+3.61*		98.75+9.64*	----

[a] Groups of 5 C57BL/6 mice were injected i.p. with a single dose of TCDD, dexamethasone or the appropriate vehicle (oil and DMSO respectively) as a control. Twelve to 120 hours later, the mice were sacrificed and mean thymic cellularity per mouse ± S.E. was calculated. The vehicle controls at various time intervals did not show significant differences. Therefore the data were pooled and depicted as a single value. Data showing statistically significant difference ($p < 0.05$) when compared to controls, have been depicted with an asterisk.

***Use of TUNEL method and flow cytometry to detect apoptotic cells*:**

Initially,we used TUNEL technique to identify the apoptotic cells which detects DNA strand breaks using TdT and FITC-dUTP. The cells were analyzed using a flow cytometer and those that exhibited green fluorescence were identified as apoptotic cells. Preliminary studies demonstrated that this method was very sensitive and also it could be used to quantify apoptosis and identify the proportion of cells that undergo apoptosis. To correlate the intensity of fluorescence with the apoptosis, we irradiated normal thymocytes from C57BL/6 mice, at 2000 rads *in vitro* and cultured them for 2, 4, 8 or 12 hours *in vitro*, in tissue culture medium and subsequently stained them with FITC-dUTP. As a control in these studies, we compared the ability of FITC-dUTP to stain normal freshly isolated thymocytes. As can be seen from Fig. 2.1, a significant but lower proportion (12.8%) of apoptotic cells could be detected as early as 2 hours of *in vitro* culture after irradiation. The percentage of apoptotic cells increased to 51.2% at 4 hours, 61.4% at 8 hours and to 70.6% at 12 hours of *in vitro* culture after irradiation. Also, it was interesting to note that the intensity of fluorescence increased with time from 2 to 12 hours of *in vitro* culture after irradiation.

For comparison, and detection of nucleosomal fragmentation of DNA, we also carried out gel electrophoresis of DNA isolated from cells in the above experiment. The data shown in Fig. 2.1 (inset), indicated that at various time points, the DNA exhibited oligonucleosomal fragmentation, characteristic of apoptotic cells. It should be noted that at 2 hours by gel electrophoresis, the bands were barely visible, whereas using flow cytometry, a significant proportion of apoptotic cells could be detected. Thus, flow cytometry, was helpful in the identification of the proportion of cells undergoing apoptosis and furthermore, the intensity of fluorescence was useful in distinguishing early and late apoptotic cells.

Studies were also conducted to address whether the percentage of apoptotic cells measured by flow cytometry would correlate with the percent fragmented DNA, using

JAM test as previously described (Matzinger, 1991; Zhu and Anasetti, 1995). To this effect, we studied the ability of dexamethasone to induce apoptosis *in vitro*. When normal thymocytes were labeled with ^{3}H-thymidine *in vitro* for 4 hours followed by incubation with dexamethasone (10μM) for 12 hours, 61% fragmented DNA was detected, which was similar to 68% apoptotic cells as detected using flow cytometry when compared to the controls (data not depicted). These results demonstrated that the percent apoptotic cells as measured byTUNEL/ flow cytometry correlates well with the percent fragmented DNA.

It should be noted that thymocytes are known to produce TdT. Therefore, studies were carried out to find out whether this would in some way interfere with the assay. These studies demonstrated that FITC-dUTP added directly to thymocytes in the absence of exogenous TdT, failed to label the cells and detect apoptosis induced by irradiation, dexamethasone (100 mg/kg) or TCDD (data not shown). Thus, endogenous TdT did not affect the assay and addition of exogenous TdT was essential to label apoptotic cells with FITC-dUTP.

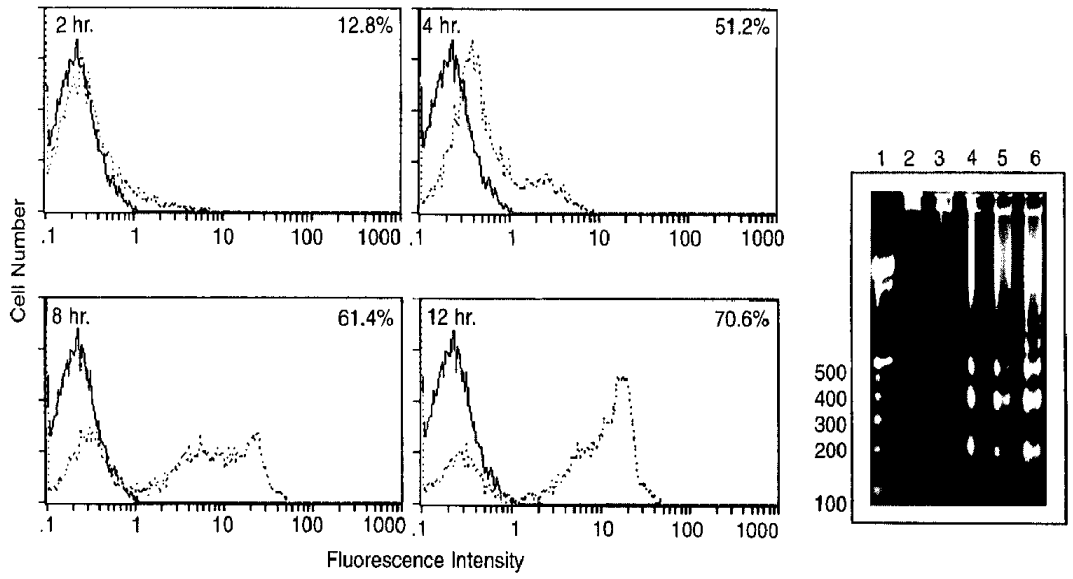

Figure 2.1: **Use of TdT and FITC-dUTP to detect apoptotic cells following irradiation**: Thymocytes from normal C57BL/6 mice were irradiated at 2000 rads *in vitro* and incubated in complete tissue culture medium for 2, 4, 8 or 12 hours at 37^0C. Next, the cells were incubated with TdT + FITC-dUTP and apoptotic cells showing green fluorescence were analyzed using a flow cytometer. The bold histogram depicts freshly isolated thymocytes from normal mice stained with TdT + FITC-dUTP and the broken histogram shows irradiated thymocytes cultured *in vitro* and similarly stained. In addition, the DNA isolated from thymocytes was also tested for nucleosomal DNA fragmentation using gel electrophoresis. In the gel, lane 1 represents a 100bp DNA marker; lane 2 normal fresh thymocytes; lane 3, irradiated cells cultured for 2 hours; lane 4, irradiated cells cultured for 4 hours; lane 5, irradiated cells cultured for 8 hours and lane 6, irradiated cells cultured for 12 hours.

***Detection of apoptosis induced by TCDD and dexamethasone in thymocytes in vivo*:**

To address whether TCDD or dexamethasone would induce apoptosis in thymocytes *in vivo,* kinetic studies were carried out at various time intervals following the administration of these compounds. TCDD or dexamethasone was administered into groups of 4-5 C57BL/6 mice and 8, 12, 24, 48, 72 and 120 hours later, the thymocytes were harvested, pooled and stained with FITC-dUTP and analyzed flow cytometrically. As shown in Fig. 2.2, administration of high dose of dexamethasone (100 mg/kg body weight) caused significant apoptosis in thymocytes and approximately 31-35% apoptotic cells could be detected as early as 8-12 hours after the drug administration. However, at 24-72 hours following high dose dexamethasone treatment, no significant numbers of apoptotic cells could be detected in the thymus of these mice. When mice were injected with 5 mg/kg body weight of dexamethasone, a similar percentage (33.1%) of apoptotic cells was seen at 12 hours. However, these cells exhibited decreased levels of fluorescence intensity when compared to thymocytes from mice treated with high dose of dexamethasone, at a similar time interval. Also, at 24-72 hours, no significant percentage of apoptotic cells could be detected.

When thymocytes from TCDD-treated mice were analyzed, it was noted that significant proportions (33-41%) of thymocytes were found to be undergoing apoptosis at 8-12 hours, whereas, no significant proportion of apoptotic cells could be detected at 24 hours or thereafter, up to 120 hours of study (Fig. 2.2). Also, the intensity of fluorescence of the apoptotic thymocytes in TCDD-treated groups was lower than that seen with thymocytes treated with higher dose of dexamethasone. However, it was similar to the thymocytes from mice treated with lower doses of dexamethasone. These data together demonstrated that apoptotic cells can be detected at early stages of the chemical administration (within 8-12 hours) but not after 24 hours, despite the fact that some treatments such as high doses of dexamethasone, caused marked thymic atrophy that persisted beyond 72 hours. The inability to detect apoptotic cells after 24 hours could have resulted from the rapid clearing of such cells by phagocytic cells (Savill *et*

al., 1993; Surh and Sprent, 1994). It should be noted that although low and high doses of dexamethasone induced similar percentages of apoptotic cells (Fig. 2.2), there was a marked difference in their effect on thymic cellularity (Table 1). This can be explained by the fact that with low dose dexamethasone treatment, the thymocytes harvested were all viable and similar in number when compared to the controls. Whereas, with the high dose dexamethasone treatment which induced a dramatic decrease in thymic cellularity, the cells analyzed represented those that had survived dexamethasone treatment. Thus, the flow cytometry data did not take into consideration majority of the cells that died and were cleared *in vivo*.

When thymocytes were investigated for apoptosis using gel electrophoresis, it was noted that only thymocytes from high dose dexamethasone-treated mice but not those from TCDD-treated or low dose dexamethasone-treated mice exhibited oligonucleosomal DNA fragmentation (Fig. 2.2, inset). In these studies, irradiated thymocytes served as a positive control and freshly isolated thymocytes as a negative control. These data demonstrated that the gel electrophoresis technique was unable to detect the early apoptotic cells that were detected using flow cytometry.

To further corroborate the data obtained using flow cytometry, thymocytes were collected 12 hours after *in vivo* TCDD-treatment and incubated with ^{3}H-thymidine *in vitro* for 4 hours and percent fragmented DNA was calculated using the JAM test. Using this assay, TCDD-treated thymocytes exhibited 58.8% DNA fragmentation when compared to the controls (data not shown). This slight increase in the percentage of DNA fragmentation when compared to the 41.4% apoptotic cells in TCDD-exposed thymocytes at 12 hours as detected using flow cytometry (Fig. 2.2), may have resulted from the extra 4 hour incubation *in vitro* with ^{3}H-thymidine in the JAM test. Thus, overall there was a good correlation between the the JAM test and flow cytometric analysis in the detection of apoptotic cells *in vivo* following TCDD treatment.

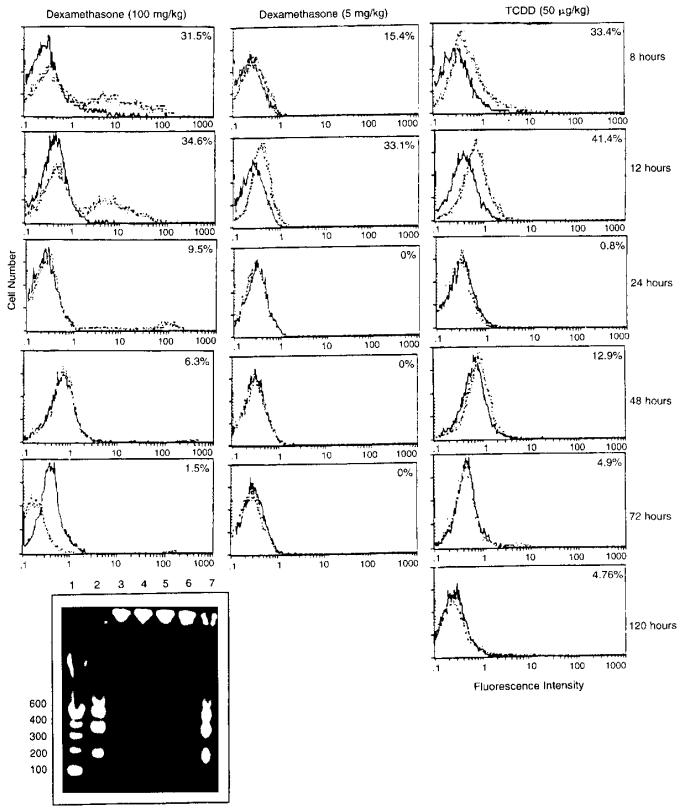

Figure 2.2: <u>Detection of apoptosis induced by TCDD or dexamethasone *in vivo*</u>: Dexamethasone, TCDD or the respective vehicle were injected into C57BL/6 mice as described in Table 1. Eight-120 hours later, the thymocytes were harvested and DNA strand breaks were detected using TdT + FITC-dUTP. The apoptotic cells showing green fluorescence were analyzed using a flow cytometer. The bold histogram depicts thymocytes from vehicle-treated controls and the broken histogram shows cells from dexamethasone or TCDD-treated mice. The subtracted histogram showing percent positive cells has been depicted. In addition, the DNA isolated from thymocytes was also tested for nucleosomal DNA fragmentation using gel electrophoresis. The thymocytes from TCDD or dexamethasone-treated mice were harvested 12 hours later and analyzed for nucleosomal DNA fragmentation using gel electrophoresis as described in Fig. 2.1. Lane 1 represents a 100bp marker; lane 2, irradiated thymocytes; lane 3, thymocytes from oil-treated controls; lane 4, thymocytes from TCDD-treated mice; lane 5, thymocytes from DMSO-treated mice; lane 6, thymocytes from low dose (5 mg/kg body weight) dexamethasone-treated mice and lane 7, thymocytes from high dose (100 mg/kg body weight) dexamethasone-treated mice.

Dose dependent induction of apoptosis by TCDD in vivo:

We next studied the effect of administration of various concentrations of TCDD on the *in vivo* induced apoptosis to determine the lowest dose at which apoptosis in thymocytes could be detected. The results shown in Fig. 2.3 indicated that when 0.1, 1, 10 and 50 μg/kg body weight of TCDD was injected into C57BL/6 mice and 12 hours later, the thymocytes were stained for apoptosis, there was a dose-dependent increase in percentage and intensity of fluorescence of apoptotic cells. Also, significant apoptosis was detected at 10 and 50 μg/kg body weight whereas little apoptosis was detected at 0.1 and 1 μg/kg body weight of TCDD.

***Thymocytes exposed to TCDD or dexamethasone in vivo and cultured in vitro exhibit increased apoptosis*:**

To overcome the problem of apoptotic cells being cleared *in vivo,* we next cultured the thymocytes in complete tissue culture medium, isolated from dexamethasone or TCDD-treated mice 12 hours after *in vivo* exposure and investigated whether such cells would exhibit increased apoptosis when compared to vehicle-treated thymocytes, incubated *in vitro* in a similar fashion. To address this, groups of 4-5 mice were injected with low dose of dexamethasone (5 mg/kg body weight) or 50 μg/kg body weight of TCDD and 12 hours later, thymocytes from these mice were isolated and cultured *in vitro* in tissue culture medium for 24 hours. The thymocytes were collected 12 hours after TCDD administration because at this time period, maximum apoptosis was detected, as shown in Fig. 2.4. Next, the cells were harvested and stained using TdT and FITC-dUTP and analyzed flow cytometrically. As a control, thymocytes were recovered from mice injected with the vehicle used for dissolving dexamethasone or TCDD.

The data depicted in Fig. 2.4 indicated that when compared to freshly isolated normal thymocytes, thymocytes from vehicle-treated groups when cultured for 24 hours *in vitro*, exhibited a significant proportion of cells undergoing apoptosis (25.2% and

23.5%, Fig. 2.4A and 2.4B). However, when thymocytes from TCDD or dexamethasone treated mice were cultured for 24 hours *in vitro*, they exhibited increased percentage of apoptotic cells (~70-75%) when compared to fresh thymocytes isolated from normal untreated mice. (Fig. 2.4C and 2.4D). In Fig. 2.4(E) and (F), we overlaid and compared the histogram of thymocytes from vehicle-treated mice with that from TCDD or dexamethasone-treated mice, cultured *in vitro* for 24 hours. As seen from these overlays, there was clearly an increase in the percentage of apoptotic cells when the thymocytes from TCDD or dexamethasone-treated mice were cultured *in vitro* (57.5% and 51.4% respectively), when compared to their respective vehicle controls, similarly cultured. These data therefore indicated that thymocytes from TCDD or dexamethasone-treated mice become increasingly susceptible to apoptosis when cultured *in vitro*.

To corroborate the findings that thymocytes exposed *in vivo* to TCDD or dexamethasone undergo increased apoptosis when cultured *in vitro*, we also analyzed such cells for DNA fragmentation using gel electrophoresis. The data shown in Fig. 2.4 (inset), confirmed these findings and the DNA from apoptotic cells exhibited the characteristic ladder formation. Lastly, we also performed the JAM test to further confirm the above findings that the *in vivo* TCDD- exposed thymocytes undergo increased apoptosis when cultured *in vitro*. To this effect, thymocytes from TCDD or vehicle-treated mice pulsed *in vitro* with ^{3}H-thymidine were cultured for an additional 24 hours *in vitro*. The percent DNA fragmentation in such cells was measured using the JAM test. These studies demonstrated that there was 55.23 % DNA fragmentation in TCDD-treated thymocytes when compared to the controls (data not depicted). It was striking that these data correlated with 57.5% apoptosis seen in TCDD-treated thymocytes when compared to the controls, as detected using TUNEL/flow cytometry (Fig 2.4E).

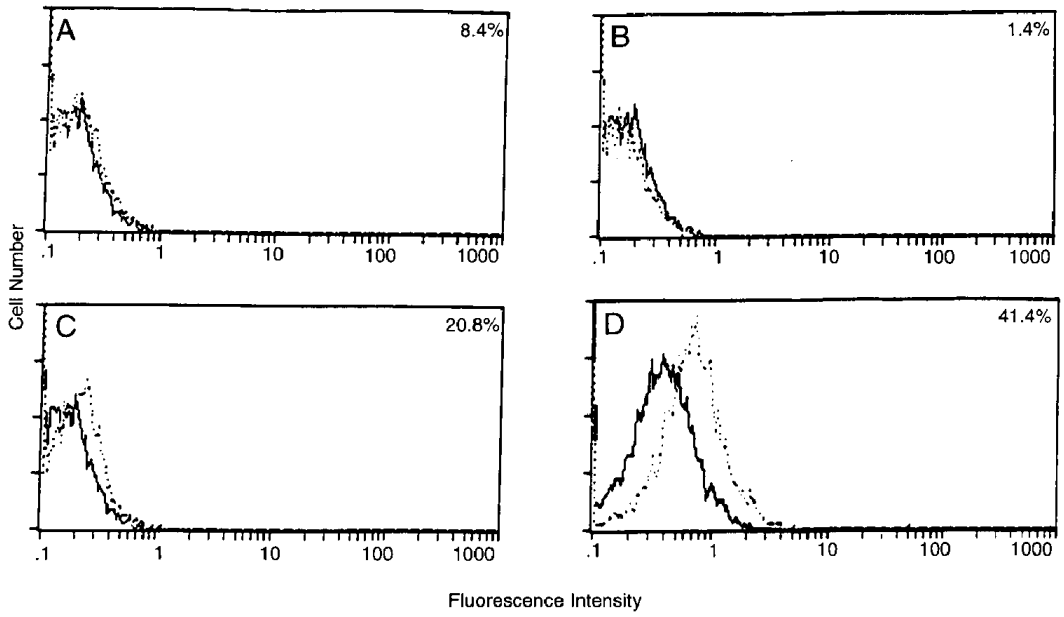

Figure 2.3: <u>Dose dependent induction of apoptosis by TCDD *in vivo*</u>: TCDD was injected into C57BL/6 mice as described in Table 1 at 0.1 (A), 1 (B), 10 (C) and 50 (D) μg/kg body weight and 12 hours later, the thymocytes were stained for apoptotic cells by flow cytometry, as described in Fig. 2.1. The bold histogram represents thymocytes from vehicle controls and broken histograms demonstrate thymocytes from TCDD-treated mice.

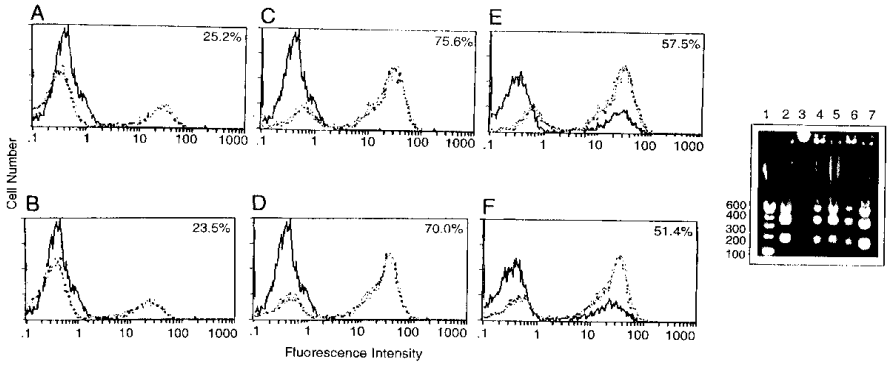

Figure 2.4: <u>Increased apoptosis of thymocytes exposed to TCDD or dexamethasone *in vivo*, upon subsequent culture *in vitro*</u>: Thymocytes were isolated from mice treated with TCDD (50 μg/kg body weight) or dexamethasone (5 mg/kg body weight) 12 hours after treatment as described in Fig. 2.2. The cells were cultured *in vitro* for 24 hours at 37^0C and the cells were analyzed for apoptotic cells using flow cytometry as described in Fig. 2.1. In (A) and (B), bold histogram represents freshly isolated normal thymocytes without *in vitro* culture and broken histogram represents thymocytes from vehicle controls for TCDD and dexamethasone respectively, cultured *in vitro* for 24 hours. In (C) and (D), bold histograms represent fresh normal thymocytes not cultured *in vitro* and broken histograms depict thymocytes from TCDD or dexamethasone-treated mice, cultured for 24 hours *in vitro*. In (E) and (F), the histogram (broken) of thymocytes from TCDD or dexamethasone-treated mice is overlaid with the histogram (bold) of thymocytes from vehicle-treated mice. In all groups, thymocytes had been cultured at 37^0C for 24 hours. The differences between broken and bold histograms is depicted as percent positive cells in each column. In addition, the DNA isolated from thymocytes was also tested for nucleosomal DNA fragmentation using gel electrophoresis. Thymocytes were isolated from TCDD or dexamethasone-treated mice 12 hours after treatment and cultured *in vitro* for 24 hours in an identical fashion as described above. The DNA extracted from such cells was analyzed for nucleosomal fragmentation using gel electrophoresis as described in Fig. 2.1. Lane 1 represents a 100bp DNA marker; lane 2, irradiated thymocytes; lane 3, fresh thymocytes; lane 4, thymocytes from oil-treated controls; lane 5, thymocytes from TCDD-treated mice; lane 6, thymocytes from DMSO-treated controls and lane 7, thymocytes from low dose (5 mg/kg) dexamethasone-treated mice.

***Addition of dexamethasone but not TCDD to the in vitro cultures of thymocytes triggers apoptosis*:**

To address directly whether TCDD causes apoptosis of thymocytes when added in culture, thymocytes from C57BL/6 mice were cultured for 2, 12 and 24 hours in the presence of TCDD, dexamethasone or the vehicle used for dissolving these compounds. After each incubation period, the cells were washed and stained with TdT and FITC-dUTP and analyzed flow cytometrically for apoptotic cells. As shown in Fig. 2.5, addition of TCDD at 1, 10 or 100 nM concentrations, failed to trigger significant apoptosis when compared to the vehicle controls. In fact, in many instances there was a decrease in the intensity of fluorescence after TCDD treatment, the exact reason for which was not clear. In contrast, when dexamethasone was added at 0.1 μM-10 μM concentration, to the *in vitro* thymocyte cultures, there was no significant induction of apoptosis at 2 hours of culture. However at 12 and 24 hours, there was clearly an increase in the percentage of apoptotic cells when compared to the vehicle controls. At 24 hours, the vehicle-treated controls also showed a significant proportion of apoptotic cells. Despite this, the addition of dexamethasone caused a further increase in the percentage of apoptotic cells. Together, these data demonstrated that only dexamethasone but not TCDD causes apoptosis when added to *in vitro* thymocyte cultures.

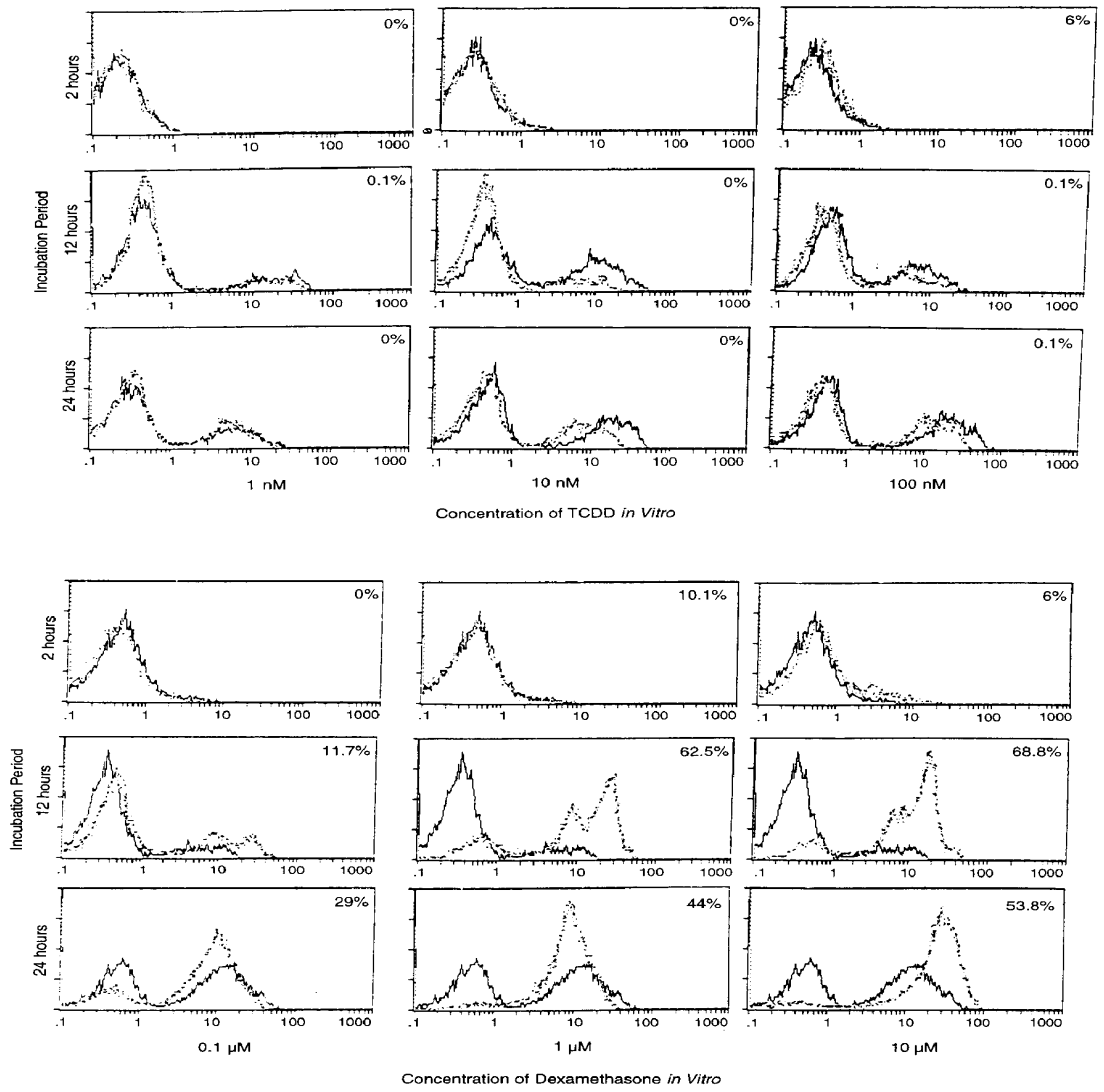

Figure 2.5: <u>Addition of dexamethasone but not TCDD to the *in vitro* cultures of thymocytes, triggers apoptosis:</u> Normal thymocytes were cultured *in vitro* for 2, 12 or 24 hours, in the presence of 1, 10, 100 nM of TCDD or the vehicle (top panel). Similarly, another set of normal thymocytes cultures were incubated with 0.1, 1 or 10 µM of dexamethasone or the vehicle (bottom panel). The cells were next analyzed for apoptotic cells using the flow cytometer as described in Fig. 2.1. The bold histogram represents thymocytes incubated with the vehicle, and broken histograms represent cells incubated with TCDD or dexamethasone. The difference between broken and bold histograms is depicted as percent positive cells in each column.

Discussion

In the current study, we demonstrated that thymocytes from TCDD-treated mice exhibited significant levels of apoptosis as detected by TUNEL method involving the ability of FITC-dUTP to label DNA strand breaks in the presence of TdT. The apoptotic cells could be detected at 8-12 hours after injection of TCDD but were not detected subsequently. Similar results were obtained with dexamethasone, especially the low dose of dexamethasone (5 mg/kg body weight). The fact that TCDD triggers apoptosis in thymocytes was confirmed using the JAM test and by the observation that *in vitro* culture of TCDD-exposed thymocytes for 24 hours, in complete tissue culture medium, resulted in increased percentage of cells undergoing apoptosis when compared to similarly cultured cells from the vehicle-treated controls. Such increased apoptosis was detected using flow cytometry, the JAM test and DNA analysis using gel electrophoresis. Together, our results suggested that the immunotoxicity of dioxin on thymocytes may be mediated through its direct ability to induce apoptosis in thymocytes.

In the current study, we made an important observation that the *in vivo* induced apoptosis can be detected only at an early stage following the administration of the drug. For example, even with high dose of dexamethasone treatment which caused a profound and long lasting atrophy of the thymus, significant *in vivo* induced apoptosis could be detected only in the first 12-24 hours and not thereafter. These data demonstrated that once the apoptosis had been induced, the dying cells were possibly cleared rapidly by the phagocytic cells, thereby making it difficult to detect apoptotic cells in the thymus. Similar observations were also made by Silverstone *et al.* (1994), suggesting that the nucleosomal length DNA fragmentation in dexamethasone-treated mice was detectable at early stages such as day 1 but not at days 2, 3, 6 and 12 post-treatment in thymocytes. Also, these investigators studied the thymocytes from TCDD-treated mice on days 3, 6, and 12 post-treatment and were unable detect DNA

nucleosomal fragmentation. However, in these studies the authors did not address whether DNA fragmentation in thymocytes could be detected at an early stage such as 12-24 hours following TCDD administration. Moreover, some studies have suggested that TCDD and dexamethasone induce differential time course of thymic involution, thereby suggesting different modes of action (Silverstone *et al.*,1994). The current study, however, suggests that time course of thymic involution induced by dexamethasone depends on the dose and that at lower doses, it's effect on the thymus is some what similar to that of TCDD.

It should be noted that the detection of nucleosomal length DNA fragmentation using gel electrophoresis is very sensitive (Silverstone *et al.*, 1994). However, the TUNEL method offers advantages such as detection of early stages of apoptosis (Gavrieli *et al.*, 1992; Gorczyca *et al.*, 1993; Kishimoto *et al.*, 1995). This may be the reason why we could detect the apoptotic cells following *in vivo* treatment using flow cytometry, in TCDD and low dose dexamethasone-treated mice, whereas, using gel electrophoresis we failed to detect the nucleosomal length DNA fragmentation. However, the latter technique was able to detect DNA fragmentation induced following high dose dexamethasone treatment *in vivo* or irradiation of thymocytes *in vitro* that triggers marked apoptosis. The fact that low intensity staining using the flow cytometric method detects early apoptotic cells was evident from the staining pattern of irradiated cells which exhibited low intensity staining at initial culture period of 2 hours and that the same cells expressed higher intensity staining with increased incubation period *in vitro*. However, using gel electrophoresis, we could barely detect DNA fragmentation at 2 hours following irradiation. For these reasons, we used in addition, the JAM test to detect DNA fragmentation in TCDD-exposed thymocytes and were able to demonstrate marked DNA fragmentation in such cells, comparable to the results obtained using TUNEL/flow cytometry.

In the thymus, a distinct population of phagocytic macrophages has been shown to clear apoptotic cells rapidly (Surh and Sprent, 1994). To overcome this problem which makes it difficult to detect apoptosis *in vivo*, we devised an alternate approach. The thymocytes were harvested within 12 hours following administration of TCDD or lower doses of dexamethasone, and were cultured *in vitro* for 24 hours in tissue culture medium to investigate whether such cells would then undergo increased apoptosis *in vitro* when compared to the vehicle-treated controls. These experiments demonstrated that thymocytes from TCDD or dexamethasone-treated animals underwent increased apoptosis when compared to vehicle-treated controls. This was demonstrable using all the three techniques used in the current study to detect apoptosis.

Previous studies have suggested that TCDD may have direct effect on the thymocytes. For example, McConkey *et al.*, (1988) demonstrated that addition of TCDD at 10 nM concentrations or higher, *in vitro*, triggered apoptosis through activation of Ca-dependent endonucleases, similar to that induced by glucocorticoids. However, other investigators have not been able to demonstrate the loss of cell viability by TCDD (Comment *et al.*, 1992). Studies carried out in our lab using both mouse and rat thymocytes have demonstrated that TCDD added at a variety of concentrations does not trigger DNA fragmentation *in vitro* as detected by gel electrophoresis (unpublished data). This was also confirmed in the current study using flow cytometric analysis. The reason why TCDD can induce apoptosis *in vivo* but not *in vitro* is not clear. It can be speculated that TCDD may trigger other cells to produce cytokines involved in apoptosis, thereby mediating this effect only *in vivo*, as discussed below.

The data presented in the current study corroborate our earlier findings that Fas^- (C57BL/6 lpr/lpr) mice were less sensitive to TCDD-mediated thymic atrophy and peripheral T cell dysfunction, when compared to the wild type Fas^+ (C57BL/6 +/+) mice (Rhile *et al.*, 1996). Although Fas is a molecule involved in apoptosis, its role in TCDD-induced immunotoxicity is not clear. It can be speculated that TCDD may

upregulate the expression of Fas or its ligand, thereby facilitating the cellular interactions in the thymocytes leading to increased apoptosis. Thymocytes undergoing apoptosis upregulate a number of cell surface markers (Kishimoto *et al.*, 1995) and moreover, TCDD can increase the serum TNF (Clark *et al.*, 1991a and 1991b), a molecule involved in thymic apoptosis (Hernandez-Caselles and Stutman, 1993). TCDD is known to be a potent transcriptional regulator of several genes in a variety of tissues inducing the cytochrome p450IA1, transforming growth factor (TGF), nuclear estrogen receptor, interleukin-1β and plasminogen activation inhibitor (Choi *et al.*, 1991; Poland and Knutson, 1982; Sutter *et al.*, 1991). Inasmuch as, cytokines such as TGF are involved in the induction of apoptosis (Rotello *et al.*, 1991), upregulation of such cytokine production may contribute towards the induction of apoptosis by TCDD.