Mechanisms of immunosuppression by organotins

-apoptosis vs. proliferative arrest-

(met een samenvatting in het Nederlands)

(con un riassunto in Italiano)

PROEFSCHRIFT

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Alessandra Gennari

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Promotor: Prof. Dr. W. Seinen
Co-promotor: Dr. R.H.H. Pieters

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Chapter one

GENERAL INTRODUCTION

ALESSANDRA GENNARI

Research Institute of Toxicology, Immunotoxicology Section, Utrecht University,
PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

1. ORGANOTIN COMPOUNDS

1.1 In general

Organotin compounds are chemicals with at least one covalent Sn-C bond. Dependent on the number of organic moieties present, they are classified as mono-, di-, tri- or tetraorganotins.

Dialkyltin compounds are widely applicated as polyvinyl chloride plastics (PVC) stabilizers and industrial catalysts, while trialkyltin compounds are used as biocides in agriculture and in anti-fouling paints in large shipping (Van der Kerk, 1978; Snoeij *et al*., 1987, Penninks *et al*., 1991). The commercial uses of organotin compounds expanded rapidly during the last 50 years: the wide application and the subsequent spread in the environment urged the need for a thorough knowledge of organotin induced-toxicity, which appeared to include hepatotoxicity, neurotoxicity, cutaneous toxicity and immunotoxicity (Snoeij *et al*., 1987).

The present thesis is mainly focused on the study of both cellular and molecular mechanisms underlying the immunotoxicity induced by organotins. The two classes of compounds that have been most extensively studied in this respect are dialkyltin homologues, such as di-*n*-butyltin dichloride (DBTC) and trialkyltin homologues, such as tri-*n*-butyltin chloride (TBTC) (Seinen *et al*., 1977a; Seinen *et al*., 1977b; Vos *et al*., 1984; Snoeij *et al*., 1988; Pieters *et al*., 1992, 1995a, 1995b; Vandebriel *et al*., 1999).

1.2 Organotin compounds and immunotoxicity

Both DBTC and TBTC are known to induce a dose-dependent and reversible reduction of thymus weight and thymocyte number in rats (Seinen and Willems, 1976; Seinen *et al*., 1977a; Snoeij *et al*., 1988). Histological investigations revealed a strong depletion of lymphocytes in the thymic cortex, without signs of overt cell destruction (Seinen and Willems, 1976; Penninks *et al*., 1985).

Phenotypical analyses of intrathymic changes after a single oral exposure of DBTC (15-20 mg/kg body weight), showed an initial selective inhibition of the number of immature proliferating thymoblasts ($CD4^-CD8^-CD2^{high}TcR\alpha\beta^{-/low}$ and $CD4^-CD8^+CD2^{high}TcR\alpha\beta^{-/low}$) within 1-2 days, resulting in a marked depletion of small cortical $CD4^+CD8^+$ thymocytes on day 4-5 after dosing (Snoeij *et al.,* 1988; Pieters *et al.*,

1992). Comparative studies of histology and kinetics of the thymus atrophy were remarkably similar for TBTC (Snoeij *et al*., 1986a). However, DBTC was found to be more effective in inducing atrophy, and further kinetic investigations have indicated that TBTC is metabolised to DBTC in the gastrointestinal tract (Snoeij *et al*., 1988).

Immature thymocyte proliferation has been shown to depend on cell contact between thymocytes and thymic epithelial cells (TEC), and this binding, mediated by adhesion molecules (Fox *et al*., 1985; Denning *et al.*, 1987), can be disturbed by DBTC (Pieters *et al*., 1995a). Being a potent thiol-reagent (Penninks and Seinen, 1983), DBTC might modify the functioning of the adhesion molecules interfering with their cytoskeletal SH-dependent organization (Geppert and Lipsky, 1991). Moreover, DBTC has been found to enhance the TcRαβ-mediated Ca^{2+} response in both thymocytes and peripheral T cells, although in case of thymocytes the increase was more pronounced. In the same study, DBTC was found to inhibit capping of TcRαβ and CD8 molecules (Pieters *et al*., 1995b).

Also TBTC had effects on cytoskeletal proteins (Marinovich *et al*., 1990a) and on the microfilament system. TBTC induced rapid depolymerization of thymocyte F-actin content, through Ca^{2+}-dependent and Ca^{2+}-independent processes (Chow and Orrenius, 1994). Particularly, trialkyltins are known to perturbate the homeostasis of calcium, inducing a rapid increase in cytosolic Ca^{2+} concentration (Chow *et al*., 1992).

Different *in vivo* studies with rats that received organotin compounds in diet for longer than 2 weeks, showed a decrease not only in thymocyte number but also in the number of peripheral T cells, as well as of T cell areas in spleen and lymph nodes (Seinen and Willems, 1976; Vos *et al*., 1984). However, the total number of B cells in the spleen was not significantly affected (Krajnc *et al*., 1984; Vos *et al*., 1990).

Parallel *in vitro* studies showed the general antiproliferative effect of DBTC and TBTC. DBTC strongly reduced the mitogen-induced proliferation of splenic T and B lymphocytes, as well as the spontaneous and mitogen-induced proliferation of thymocytes (Seinen *et al*., 1979), and TBTC concentration-dependently reduced the number of bone marrow cells (Snoeij *et al*., 1986b).

1.3 Cellular and biochemical toxicity of organotin compounds

The cytotoxic effects of organotin compounds have been intensively investigated. Diorganotin compounds inhibit the α-keto acid oxidizing enzyme complexes in mitochondria. Since they have a high chemical affinity for dithiol groups, this inhibition was suggested to be due to binding to lipoic acid or lypoyl dehydrogenase (Aldridge, 1976; Penninks and Seinen, 1980). At higher concentrations, DBTC was also found to inhibit the oxidative phosphorylation processes in mitochondria (Cain *et al*., 1977; Penninks *et al*., 1983). Dialkyltin can also affect cellular metabolism, provoking an increase in the consumption of glucose and accumulation of pyruvate and lactate, but without affecting the intracellular ATP levels in glucose-containing media (Penninks and Seinen, 1980).

Triorganotins are effective inhibitors of mitochondrial ATP synthesis (Aldridge, 1976; Snoeij *et al*., 1987). They mediate an exchange of hydroxyl ions across the mitochondrial membranes, resulting in a disturbance of the existing proton gradient, and they bind to a component of the ATP synthase complex, leading to an inhibition of ATP production. Studies on cellular effects indicated an increase in the consumption of glucose and accumulation of lactate, and a marked reduction of ATP level (Snoeij *et al*., 1986c).

Possibly related to the above-mentioned cytotoxic effects, organotin compounds interfere with macromolecular synthesis (DNA, RNA and protein) in rat thymocytes. The incorporation of thymidine into DNA and of L-proline or L-leucine into protein is decreased by tributyltin and dibutyltin compounds, while the incorporation of uridine into RNA is stimulated. For TBTC it has been demonstrated that this increase of RNA occurs particularly in small thymocytes (Penninks and Seinen, 1983; Snoeij *et al*., 1986c).

2. APOPTOSIS

2.1 In general

Necrosis and apoptosis are two distinct modes of cell death (Fig. 1). During necrosis the permeability of the plasma membrane increases, the endoplasmic reticulum dilates, the cell alters shape with blebbing of the surface, the mitochondria become denser, the nuclear chromatin flocculates, and cell releases lysosomal enzymes that may provoke an inflammation reaction.

A cell undergoing apoptosis rounds up, severing junctions with its neighbours and losing microvilli. The cytoplasm condenses, but the plasma membrane, ribosomes, Golgi apparatus, and mitochondria remain intact. The endoplasmic reticulum dilates, forming vesicles that fuse with the surface membrane giving a characteristic bubbling appearance. The chromatin condenses at the nuclear membrane and the cell separates into apoptotic bodies that are recognized, phagocytosed and digested by macrophages or epithelial cells. In this way dead cells are rapidly removed, and any leakage of their possibly dangerous contents is avoided.

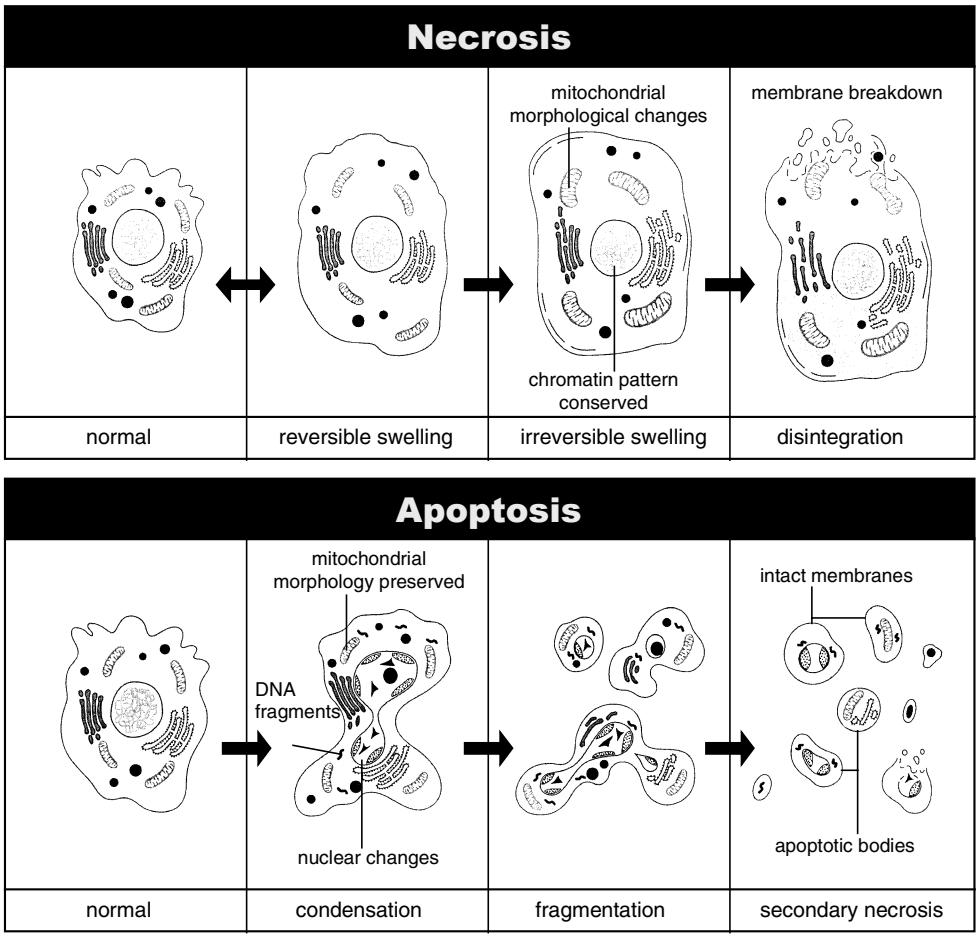

Fig. 1. Morphologic characteristics of necrosis and apoptosis, two distinct patterns of cell death. Adapted from Duvall and Wyllie (1986).

Apoptosis is the mechanism of cell death underlying programmed cell death during embryogenesis, and as such crucial for maintenance of tissue shape and cell renewal. In the immune system, apoptosis is an important homeostatic process in normal tissue physiology, where it mediates the silencing of autoreactive precursor cells during T and B cell development and eliminates expanded clones at the termination of an immune response. Regulation of apoptosis involves signal transduction networks as complex as those regulating cell proliferation and differentiation (McConkey *et al*., 1994). Defects in the control of apoptosis have been linked to various diseases, including autoimmunity (Ravirajan, *et al.,* 1999), neurodegenerative diseases (Raff *et al.,* 1993), and acquired immunodeficiency syndrome (AIDS) (Gougeon and Montagnier, 1993).

2.2 Nuclear changes

In the nucleus, the chromatin is condensed at the nuclear membrane and then degraded into fragments, all integer multiples of about 190 base pairs of DNA (Wyllie and Morris, 1982). This pattern of cleavage is due to the vulnerability of the linker DNA between nucleosomes to various Mg^{2+} and Ca^{2+}-dependent endonucleases. DNA fragmentation is a very important hallmark of apoptosis, and can be made visible on agarose gels (so-called DNA laddering).

2.3 Ca^{2+} and cytoskeleton alterations

Normal cytoskeletal organization is essential for several cellular processes. Disruption of this organization results in the appearance of surface protrusions known as blebs, which can rupture, compromising plasma membrane integrity. The involvement of Ca^{2+} in the appearance of surface abnormalities has been suggested by the observation that blebs occur when cells are treated with toxic agents that cause a sustained increase in cytosolic Ca^{2+} level. Ca^{2+} is also involved in the interactions between the various structural components of the cytoskeleton, via Ca^{2+}-binding proteins and Ca^{2+}-dependent enzymes (Orrenius *et al*., 1989). In particular, the association of the cytoskeletal network with the plasma membrane and the interaction of different classes of cytoskeletal fiber appear to be strictly dependent on the cytosolic Ca^{2+} level. An increase in cytosolic Ca^{2+} concentration causes the cleavage of actin microfilaments from α-actinin, disturbing the

association with the plasma membrane and leading to the production of surface blebs, which is one of the characteristic morphological alterations during apoptosis.

Ca^{2+} has been shown to mediate endonuclease activation, which results in the cleavage of target-cell chromatin into oligonucleosome fragments. Intracellular calcium chelators and extracellular EGTA can inhibit DNA fragmentation and apoptotic cell death, suggesting that sustained Ca^{2+} elevations are required for both responses (McConkey *et al*., 1989; *Aw et al*., 1990).

2.4 Cell surface alterations

It is characteristic of apoptotic cells that they are rapidly recognized and phagocytosed by their neighbours or macrophages. It seems that recognition of apoptotic cells involves existing specific receptors on acceptor cells binding to newly exposed ligands on apoptotic cells (Arends and Wyllie, 1991). One common parameter for detection of apoptosis is the presence of phosphatidyl serines (Fadok *et al.,* 1992), which are normally located inside the plasma membrane, at the extracellular side.

2.5 Macromolecular synthesis

Several studies showed that apoptosis is an active, energy-requiring process, and that the synthesis of a new protein or proteins is most likely required for programmed cell death to occur. This has been confirmed by the finding that apoptosis can be prevented by inhibitors of synthesis of RNA (actinomycin D) and protein (cycloheximide) in different cell types and circumstances (Wyllie *et al.,* 1984; Compton *et al.,* 1988; Martin *et al*., 1988; Lanotte *et al*., 1991).

2.6 Mitochondria-cytochrome c

Numerous recent finding suggest that mitochondria have a central role in the apoptotic process (Marchetti *et al.,* 1996; Kroemer, 1997a). Two different major changes in membrane permeability have been observed. Firstly, the electrochemical gradient built up over the mitochondrial inner membrane dissipates during apoptosis (Zamzami *et al.,* 1995). Secondly, proteins that normally are sequestered in mitochondria are released through the outer mitochondrial membrane to the cytosol. Such proteins include

cytochrome *c,* which functions as an electron carrier in the respiratory chain (Liu *et al.,* 1996).

The exact molecular mechanisms and the cause/effect relationship between the increase in inner and outer mitochondrial membrane permeability are still not completely clear. It has been hypothesized that opening of the so-called mitochondrial permeability transition pore (PT), which is formed by proteins within the contact site of the inner and outer membranes, might be closely linked to both the dissipation of the inner transmembrane potential ($\Delta\Psi$) and the cytochrome *c* release (Kroemer, 1997b).

2.7 Reactive oxygen species (ROS)

As the terminal electron acceptor for oxidative phosphorylation, molecular oxygen occupies an essential role in many of the metabolic processes associated with an aerobic existence. However, this thirst for electrons also leads to the formation of a variety of reactive oxygen intermediates, oxygen species that have either unpaired electrons (i.e. O^{-}_{2}, OH•) or the ability to abstract electrons from other molecules (i.e. H_2O_2) (Halliwell and Gutteridge, 1990). Reactive species react with cellular macromolecules, damaging them directly or indirectly. Consequences of ROS toxicity include protein alterations, nucleic acid damage or oxidation of membrane lipids, which result in the loss of plasma membrane integrity. Mitochondria are believed to be an important source for ROS production (Corsini *et al*., 1998). It has been shown that many of the chemicals capable of inducing apoptosis are also associated with oxidative stress, suggesting an active role for reactive oxygen species in cell death (Buttke and Sandstrom, 1994).

2.8 Caspase family

Overwhelming evidence suggests the involvement of specific cysteine aspartate-specific proteases (caspases) in apoptosis (Kumar and Lavin, 1996; Zhivotovsky *et al.,* 1997). Caspases catalyse a highly selective pattern of protein degradation, and are all synthesised as proenzymes that are proteolytically processed to form active enzymes. The cleavage sites for proteolytic maturation of procaspases are themselves cleaved by caspases, suggesting that caspases may engage in a cascade of activation and

amplification steps (Hirata *et al.,* 1998). Different proteins, with an important role in causing cell death, can be digested by caspases (Penninger and Kroemer, 1998).

At least 14 different caspases have been identified in man (Van de Crean *et al.,* 1998), but in particular the subfamily of caspase 3-like proteases, including caspase 3, 6, 7 and 9, seems to be involved in apoptosis. These enzymes mediate apoptosis by cleaving selected intracellular proteins such as proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmatic reticulum, and cytosol (Kumar and Lavin, 1996). Furthermore, the activation of caspase 3 seems to be preceded by the release of mitochondrial cytochrome *c* (Bossy-Wetzel *et al.,* 1998).

3. ORGANOTIN COMPOUNDS AND APOPTOSIS

Physiologic triggers are not the only ones capable of inducing apoptosis in susceptible target cells, and many chemicals exert their effects by stimulating apoptosis by exploiting physiologic control mechanisms.

The combination of cytotoxic effects of DBTC and TBTC (Cain *et al*., 1977; Snoeij *et al.,* 1987; Marinovich *et al.*, 1990b) have suggested that organotins might induce apoptosis in thymocytes and possibly other cells, and in fact this has been demonstrated to be the case for TBT-compounds. DBTC appeared unable to significantly increase DNA fragmentation *in vitro*, although only one concentration at one time point was tested (Aw e*t al*., 1990; Raffray and Cohen, 1993).

It has been shown that the interaction between micromolar concentrations of TBTC and the thymocyte plasma membrane causes the opening of Ca^{2+} channels, which leads to a sustained elevation of cytosolic Ca^{2+}. The latter stimulates an endogenous endonuclease activity and directs thymocytes into apoptosis (Aw *et al*., 1990; Chow *et al*., 1992). More recently, Corsini *et al.* (1998) demonstrated in murine keratinocytes that TBTC induced ROS production at the mitochondrial level, which is preceded by alterations of Ca^{2+} homeostasis. Furthermore, targeting of mitochondria by TBTC has been shown capable to release pro-apoptotic factors, such as cytochrome *c*, considered as a primary event in the induction of DNA fragmentation (Liu *et al*., 1996; Kroemer, 1997). TBTC induced the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol

in Jurkat cells and subsequently activated caspase 3, which leads to the irreversible damage of the cell (Stridh *et al*., 1998).

In vivo experiments showed that apoptotic cell death occurs in the thymus after exposure to relatively high doses of tributyltin-oxide (TBTO) (Raffray and Cohen, 1993).

4. AIM OF THE THESIS

Over the past 10 to 20 years many attempts have been made to identify the mechanism involved in organotin-induced thymus atrophy (Krajnc *et al*., 1984; Vos *et al*., 1984; Penninks *et al.*, 1985, 1991; Snoeij *et al*., 1987, 1988; Marinovich *et al.,* 1990a; Pieters *et al*., 1992, 1995a, 1995b). Although considerable progress has been made, the apparent discrepancy between the thymus selectivity *in vivo,* and the general antiproliferative effect *in vitro,* has hampered inclusion of specific *in vitro* data in a mechanism that might explain the *in vivo* observations. The absence of any sign of apoptosis *in vivo* after exposure to selective anti-proliferative doses of organotins, and the findings indicating that TBT-compounds are rapidly metabolised into DBT-compounds, are considered as additional confusing factors. The aim of this project was therefore to further elucidate the mechanisms underlying thymus atrophy induced by the organotin compounds TBTC and DBTC by focusing on the following two issues:

- Apoptosis in relation to dose and concentration: has apoptotic cell death a role in the thymus at antiproliferative doses? Is DBTC also able to induce apoptosis *in vitro*? Is there a similarity in the mechanism of apoptosis between TBTC and DBTC?
- Thymus selectivity: is the thymus sensitive because it is a highly proliferative organ or do organotins have a wider anti-proliferative effect?

The second chapter evaluates the importance of DNA fragmentation in thymus atrophy induced by organotin compounds, at doses known to specifically affect thymocyte proliferation. The finding that apoptosis is not relevant *in vivo* at anti-proliferative doses of DBTC or TBTC, but may be so at higher doses, pushed us to focus on *in vitro* induced-apoptosis by organotins, in order to elucidate the molecular (RNA and protein) and cellular (calcium, mitochondria, cytochrome *c* and caspases) mechanisms involved in this kind of cell death (chapter 3 and 5). In chapter 4, the use of a recently developed

technique, called cDNA array assay, helped us to visualize some of the genes up regulated by organotin compounds during *in vitro* apoptosis. Finally, an *in vivo* study is performed to evaluate whether anti-proliferative organotin compounds are really thymus-specific or rather have a generally antiproliferative and immunosuppressive effect (chapter 6). Chapter 7 summarizes and discusses the contents of this thesis.

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Chapter two

ORGANOTIN-INDUCED APOPTOSIS AS OBSERVED *IN VITRO* IS NOT RELEVANT FOR INDUCTION OF THYMUS ATROPHY AT ANTI-PROLIFERATIVE DOSES

ALESSANDRA GENNARI, MARC POTTERS, WILLEM SEINEN, AND RAYMOND PIETERS

Research Institute of Toxicology, Immunotoxicology Section, Utrecht University, PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

ABSTRACT

The organotin compounds di-*n*-butyltin dichloride (DBTC) and tri-*n*-butyltin chloride (TBTC) selectively cause thymus atrophy. Previously, DBTC and TBTC were shown to inhibit proliferation of immature thymocytes, but other studies demonstrated that TBTC but not DBTC increased apoptosis *in vitro* and also *in vivo*. In this study we examined whether apoptosis is increased *in vitro* by DBTC and TBTC at various concentrations and periods of incubation, and whether apoptosis is involved in the induction of thymus atrophy at selective antiproliferative doses. *In vitro*, DBTC or TBTC at a concentration of 3 µM significantly increased DNA fragmentation in freshly isolated rat thymocytes after preincubation for 10 min followed by a 22-hr culture period. After continuous exposure for 22 hr, apoptosis was observed to be optimum at 1 µM TBTC and 0.3 µM DBTC and lower or absent at higher concentrations. Apparently, apoptosis induced by organotins *in vitro* depends on both duration and concentration of exposure. Selective antiproliferative doses of DBTC nor TBTC increased apoptosis on day 1 or 2 after single oral exposure. In contrast, the corticosteroid dexamethasone caused a depletion of both small and large thymocytes and a marked increase of apoptosis on both days 1 and 2 after dosing. Thus, although apoptosis is involved in the *in vitro* cytotoxic effects of both organotin compounds, it seems not involved in thymus atrophy at a dose that selectively inhibits immature thymocyte proliferation.

INTRODUCTION

Organotin compounds are in use in various industrial and agricultural applications. Derivatives of dialkyltin compounds like di-*n*-butyltin dichloride (DBTC) are in use as PVC stabilizers and industrial catalysts, whereas trialkyltin compounds, such as tri-*n*-butyltin chloride (TBTC), are potent biocides in crop protection and anti-fouling paints for large shipping (Fent, 1996).

Both organotins cause thymus atrophy and, after prolonged exposure, suppress T-cell-mediated immune responses (Seinen and Willems, 1976; Seinen *et al*., 1977, 1979; Vos *et al.,* 1984). Previous kinetic studies have indicated that TBTC is dealkylated to DBTC and that DBTC is the toxicologically active compound (Snoeij *et al.*, 1988). Subsequent mechanistic studies have therefore focussed on the thymic effects of DBTC as the most

suitable thymotoxic model organotin compound. After single oral exposure to DBTC (15 mg/kg) a maximum depletion of the large population (80-85% of all thymocytes) of small cortical $CD4^+CD8^+$ thymocytes occurred 4 to 5 days later (Pieters *et al*., 1992). Importantly, this depletion was preceded by a maximum decrease in the number of immature proliferating thymoblasts at 24-48 hr after dosing. Additional phenotypical analyses of the early thymic effects in combination with the fast turnover of the $CD4^+CD8^+$ thymocyte population, suggest that organotin-induced thymus atrophy is initiated by selective inhibition of the proliferation of immature $CD4^-CD8^+$ thymocytes. This population constitutes a highly proliferative subset in transcience between the very immature $CD4^-CD8^-$ $TcR\alpha\beta^{-/low}$ and the more mature $CD4^+CD8^+TcR\alpha\beta^{-/low}$ population (Snoeij *et al.,* 1988; Pieters *et al.,* 1993).

Recently, it has been shown that increased apoptotic cell death may be an alternative or additional cause in TBTC-induced thymus atrophy (Raffray and Cohen, 1993). DBTC was not tested *in vivo*, but appeared unable to induce a substantial increase in apoptosis *in vitro*, although only one concentration at one time point was tested (Aw *et al*., 1990; Raffray and Cohen, 1993). As TBTC is likely to be dealkylated to DBTC *in vivo* and because of our previous findings that inhibition of proliferation underlies organotin-induced thymus atrophy, we investigated whether DBTC or TBTC induces apoptosis at selective antiproliferative doses. Moreover, as cytotoxicity of TBTC depends on the concentration and duration of exposure (Zucker *et al*., 1992), we set out to determine if DBTC increases DNA fragmentation *in vitro* at varying concentrations and/or time periods of incubations.

MATERIALS AND METHODS

Chemicals and solutions

DBTC was obtained from Johnson Matthay Alfa Products (Karlsruhe, Germany), TBTC was a gift from Dr. H. Meinema (TNO, Zeist, The Netherlands) and dexamethasone (DEX) was purchased from Sigma (St. Louis, MO). Propidium Iodide (5 µg/ml PI, Sigma) was dissolved in PBS. Ribonuclease A (RNase, type I-A, Sigma) was dissolved first in Tris buffer (10 mg/ml), boiled for 15 min, and then diluted in RNase buffer (0.1% sodium citrate, 0.1% Triton X-100) to obtain a final concentration of 0.5 mg/ml.

5-Bromo-4-indolylphosphate (BCIP, Boehringer Mannheim, B.V., Almere, The Netherlands) was dissolved in N,N-dimethylformamide and nitroblue tetrazolium (NBT, Sigma) was dissolved in bidest. RPMI-1640 (Gibco, Paisley, UK) was supplemented with 5% heat-inactivated fetal calf serum (Gibco), 1% glutamine (Gibco), penicillin/ streptomycin (Flow, Irvine, UK), and sodium bicarbonate (Gibco), 1% Hepes buffer (Gibco). PBS was supplemented with 4 mM Ca^{2+} and 10 mM Mg^{2+} and when used for preparation of cell suspensions also with 2 mM glucose.

Animals and treatment

Male Wistar-derived rats (U:Wu) were bred under specific pathogen-free conditions and housed under standard conditions (room temperature ±20℃, relative humidity ±55%) in the breeding facilities of the Utrecht University (Utrecht, The Netherlands). Rats were 4-5 weeks of age at the start of the experiments, because at that age their thymus is optimal of size and effects of organotins are most pronounced compared to controls (Seinen and Willems, 1976; Snoeij *et al.*, 1988; Pieters *et al.*, 1992).

For the *in vivo* studies, animals (four per group) were intubated with a dose of 20 mg DBTC or TBTC/kg body wt. Previously, this dose was shown to cause thymus weight reduction to about 50% of control (Snoeij *et al.,* 1988). The organotins were dissolved in ethanol absolute (Merck, Darmstadt, Germany) and further diluted in corn oil (250 ml per rat) to a final ethanol concentration of 5% (v/v). Dexamethasone (3 mg/kg body wt) was dissolved in saline and given intraperitoneally. Control rats were intubated with the same volume of corn oil (250 µl, 5% ethanol) without organotin compounds. Rats were killed by decapitation and thymi were isolated. Half of the thymus was then snap-frozen in liquid N_2 and the other part was used to prepare cell suspensions.

Cell suspensions and incubation

Cell suspensions from thymi and spleens were prepared by crushing the organs on a nylon sieve and collecting the cells in ice-cold PBS. After centrifugation, cells were resuspended in RPMI-1640 or, for the thymocytes obtained from the *in vivo* treated rats, in PBS and counted by a Coulter counter ZM (Coulter Electronics, Luton, UK). In case of thymocytes, numbers of total cells (diameter>4.3 µM) and large cells (diameter>8.2 µM),

representing proliferating thymoblasts, were determined as described before (Snoeij *et al.*, 1988) by varying the threshold values on the Coulter counter.

For *in vitro* incubations, DBTC, TBTC, and DEX (final concentration, 10 µM) were dissolved in ethanol absolute and then diluted in RPMI. The final ethanol concentration was 0.1%, which was ineffective by itself. Cells were incubated for 10 min, washed twice, and incubated overnight (about 22 hr) without the compounds or were incubated continuously for 22 hr with varying concentrations of organotins at 37°C. DEX was present throughout the whole incubation period of 22 hr.

Detection of apoptosis

By flow cytometry. Detection of DNA fragmentation by flow cytometry with PI as fluorescent indicator was done essentially as described by Nicoletti *et al*. (1991). Briefly, after incubation with the test compounds, $5x10^6$ cells/ml were centrifuged and resuspended in 1 ml PBS. Of this cell suspension 200 µl was incubated for 30 min with 0.5 ml RNase (0.5 mg/ml) at room temperature. Then, 0.5 ml PI was added (5 µg/ml) and the fluorescence of individual nuclei was measured (in the FL_2 channel set for log scale) on a FACScan flow cytometer (Becton-Dickinson, Erembodegem, Belgium).

By in situ labeling. The immunohistochemical *in situ* cell death detection kit of Boehringer Mannheim was used essentially according to the manufacturer's instructions. Briefly, frozen tissue sections were fixed with 4% paraformaldehyde, washed with PBS, and incubated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate). After washing, 50 µl TUNEL reaction mixture (label solution + enzyme solution) was first added on each section followed by 50 µl of converter-AP (alkaline phosphatase). Finally, the substrate solution (18 mg/ml BCIP, 5 mg/ml NBT, Tris/HCl/Mg buffer) was added and the labeled DNA strandbreaks were analyzed by light microscopy.

Statistics

One-way ANOVA with Scheffe's post hoc test was used to assess significance of differences between group means. p values < 0.05 were considered significant.

RESULTS

DBTC and TBTC increase apoptosis in vitro

Freshly isolated rat thymocytes kept overnight at 37℃ without further treatment already showed an increase of DNA fragmentation when compared to thymocytes stored overnight at 4℃ (data not shown). The extent of fragmentation at 37℃ was the same after incubation with the solvent ethanol (0.1%, v/v) and considered background. After incubation for 10 min with either DBTC or TBTC followed by two washes, the percentage of apoptotic nuclei was increased after a culture period of 22 hr (Fig. 1A). This effect appeared dose-dependent but was only statistically significant at 3 µM for both organotins. After continuous exposure for 22 hr, an optimum in DNA fragmentation was observed at 1 µM for TBTC and 0.3 µM for DBTC (Fig. 1B). At 1 µM DBTC DNA fragmentation was at background level whereas at 3 µM DBTC the percentage of DNA fragments was as low as the 0℃ control.

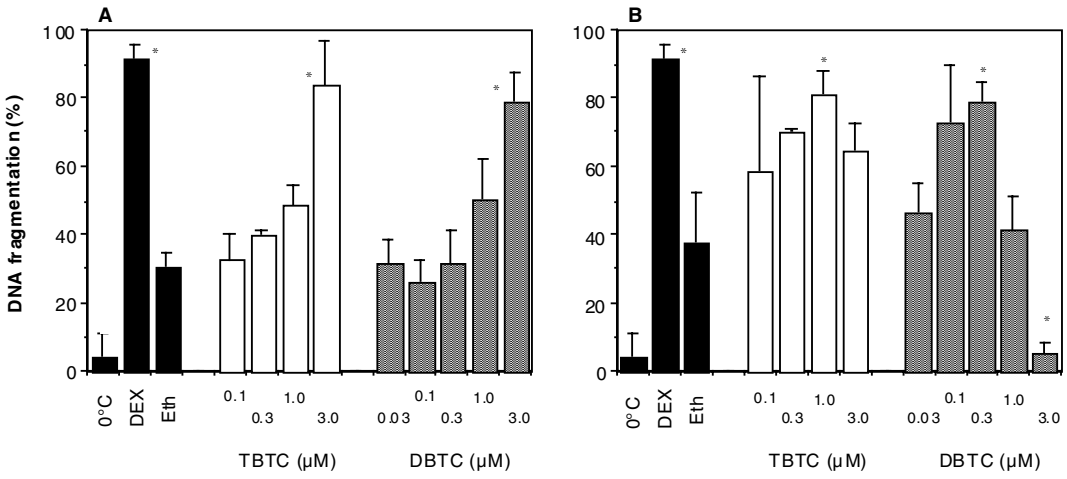

Fig. 1. Relative DNA fragmentation in thymocytes incubated *in vitro* with graded concentrations of DBTC or TBTC as measured by flow cytometry. 0℃, DEX, and ethanol served as controls. (A) 10-min preincubation; (B) 22-hr continuous exposure. *p<0.05.

Organotins have been shown to selectively decrease thymus weight, without affecting spleen weight or morphology (Vos *et al.,* 1984; Snoeij *et al.,* 1988). To assess whether the apoptotic effects of organotins were selective *in vitro* as well, we have tested whether

organotin induces apoptosis in spleen cells. As shown in Fig. 2, spleen cells treated for 10 min with DBTC or TBTC and then cultured 22 hr without compounds demonstrated DNA fragmentation.

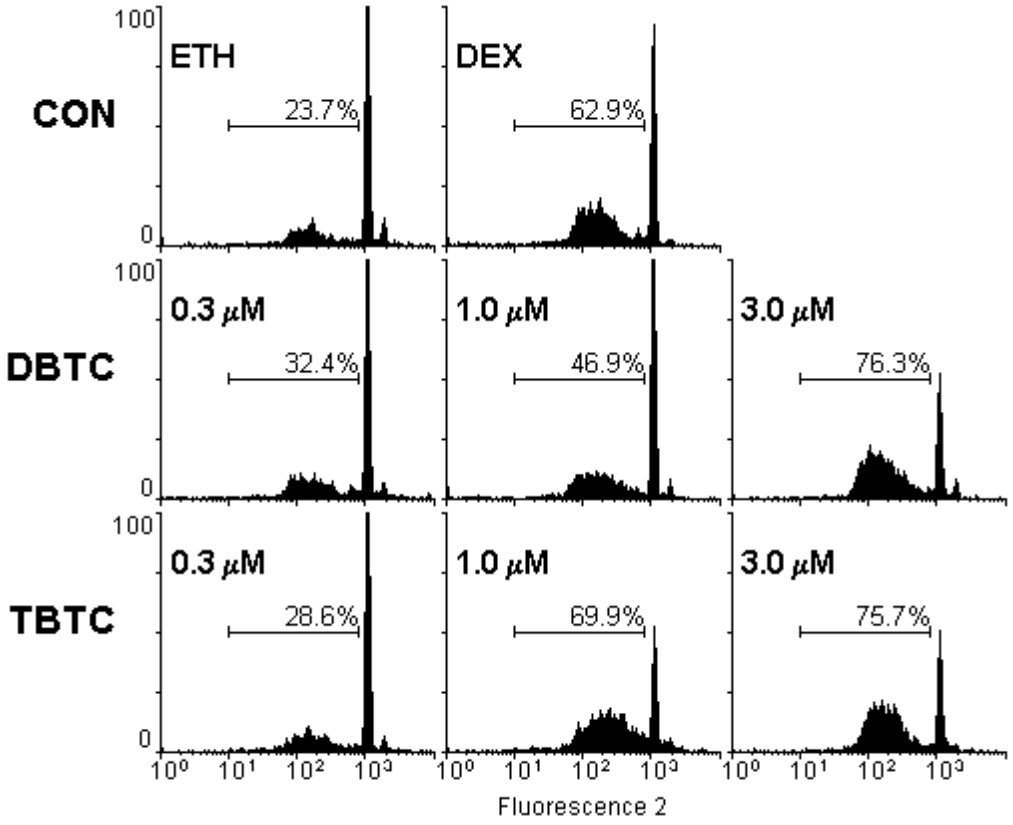

Fig. 2. Relative frequency of DNA fragmentation in spleen cells *in vitro* induced by the organotin compounds DBTC or TBTC after a 10-min preincubation. DEX and ethanol served as controls. Histograms are from one representative experiment out of three.

DBTC and TBTC do not induce apoptosis in vivo

One day after injection of the positive control DEX (3 mg/kg), ±22 % of the DNA collected from thymi was fragmented as assessed by flow cytometry (Fig. 3). By immunohistology a massive increase of apoptotic cells was demonstrated in the thymus cortex (Fig. 4). On day 2 after dosing, the percentage of apoptosis was reduced (data not shown), which may be due to phagocytic activity of reticular cells or macrophages in the thymus (Boyd *et al*., 1993).

In contrast, no DNA fragmentation was detected by flow cytometry (Fig. 3) and no increase of apototic cells was detected *in situ* (Fig. 4) on any of the 2 days after oral treatment with DBTC or TBTC.

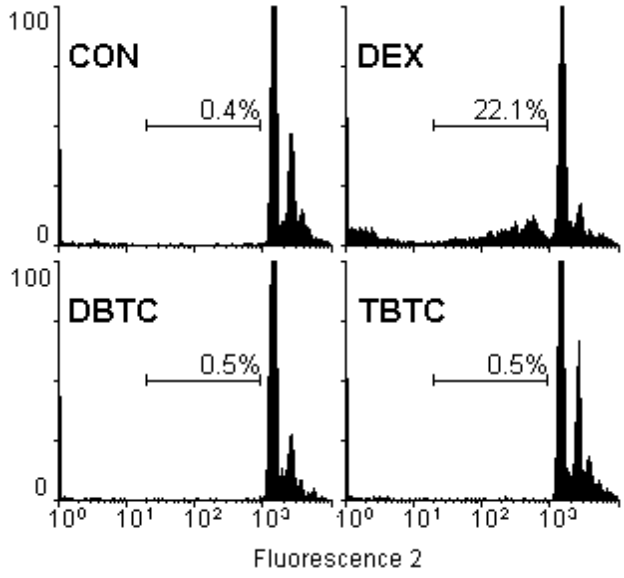

Fig. 3. Flow cytometry of PI-stained nuclei of thymocytes obtained from rats treated with corn oil (CON), DEX, DBTC or TBTC, 24 hr after dosing. Histograms are from one representative animal of four per treatment.

Organotin compounds, unlike DEX, selectively decrease the number of large thymocytes

On day 1, the number of both total and large cells was profoundly reduced in DEX-treated rats when compared to controls or organotin-treated rats. Organotin treatment did not affect the number of total cells, whereas the number of large cells tended to decrease compared to controls (Table 1).

On day 2, absolute thymocyte numbers in control animals seemed increased compared to day 1, but this was not significant and possibly the result of the still growing thymus in these young rats. On the same day, organotins caused a decrease in total number of thymocytes to around 50-60% of control, whereas the number of large cells was around 30% of control. DEX was less selective as it reduced the number of total and large thymocytes to around 9 and 12 % of control, respectively (Table 1).

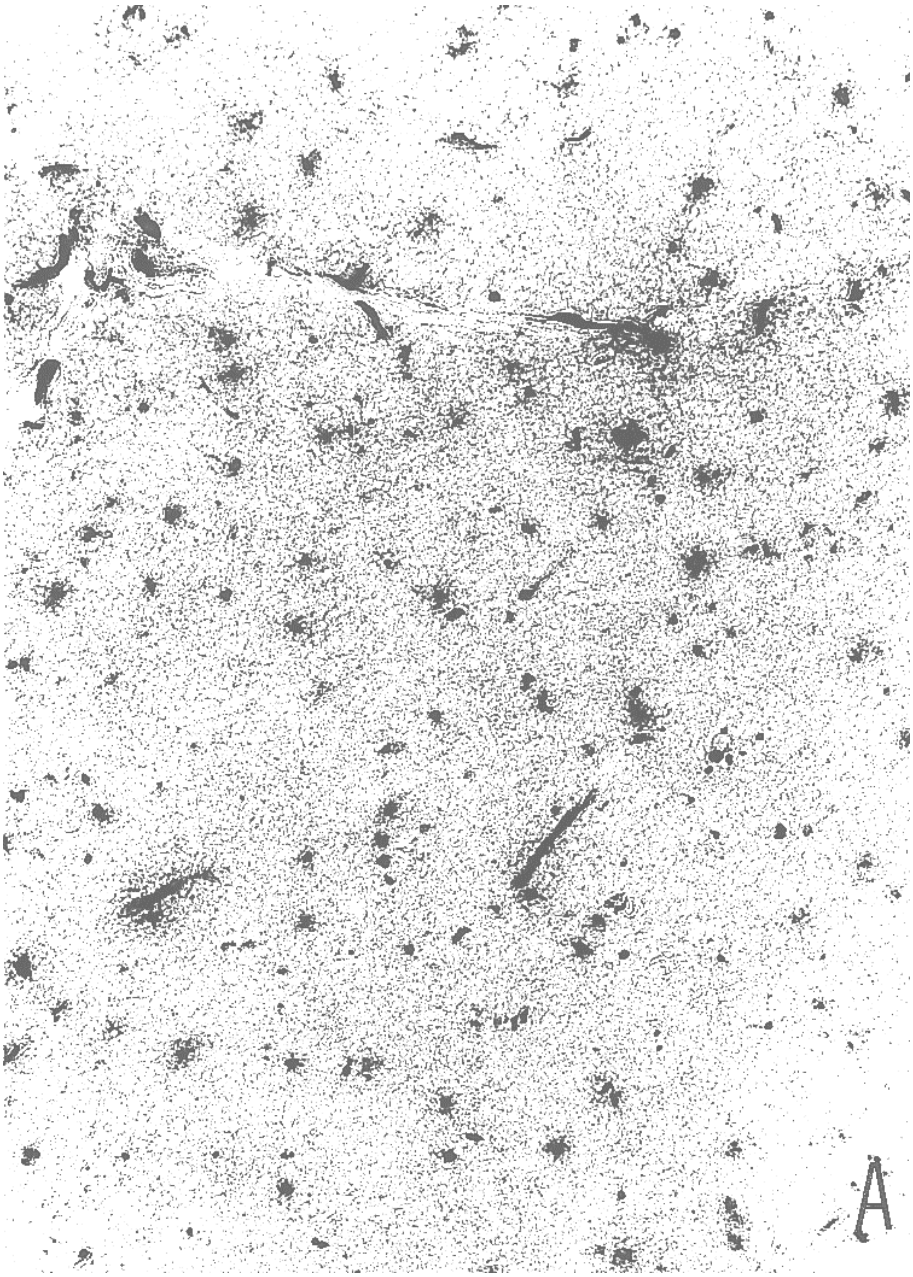

Fig. 4. Immunohistochemical staining for apoptosis on thymus sections obtained from control rats (A), or DBTC (B)-, TBTC (C)- or DEX (D)-exposed rats, 24 hr after dosing. Magnification 200x.

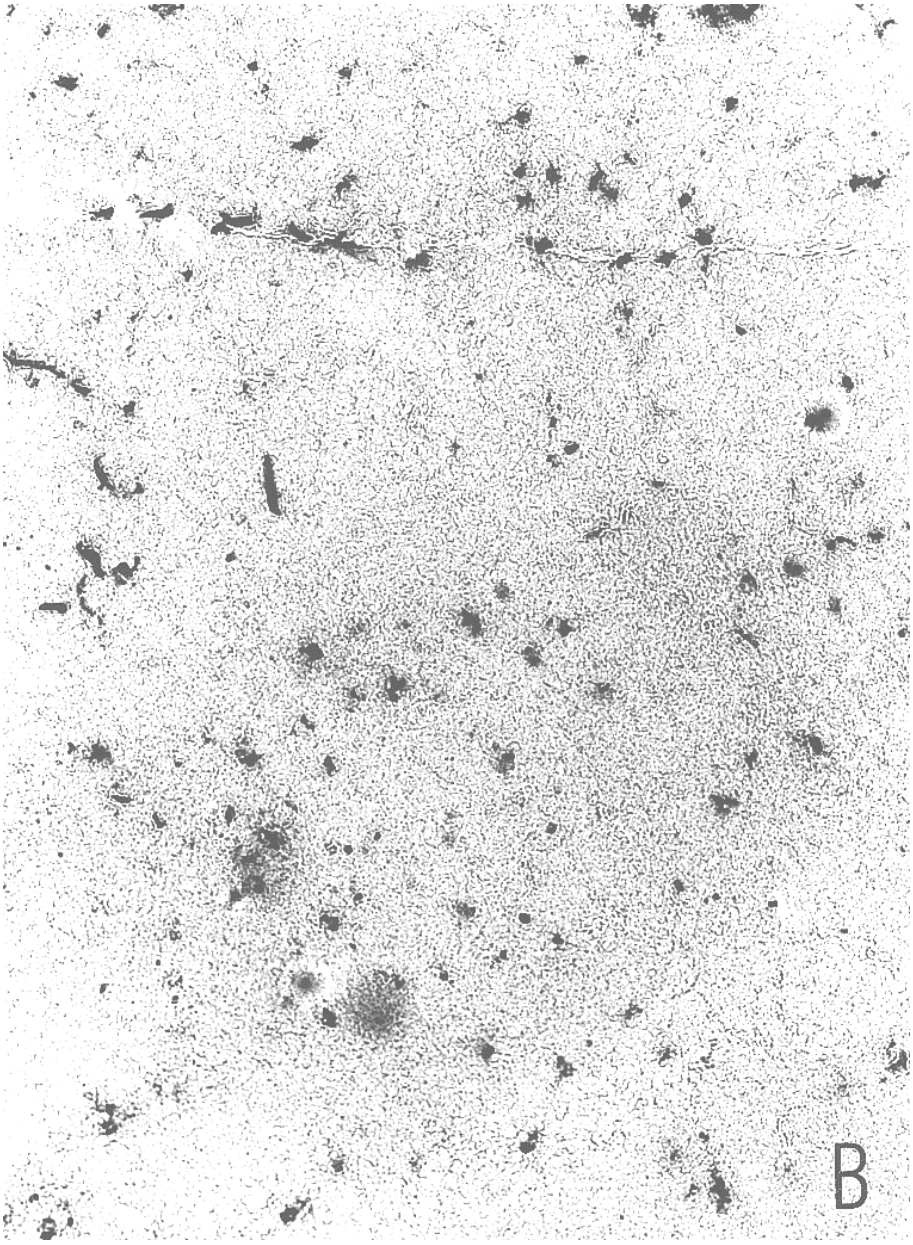

Fig. 4. - continued

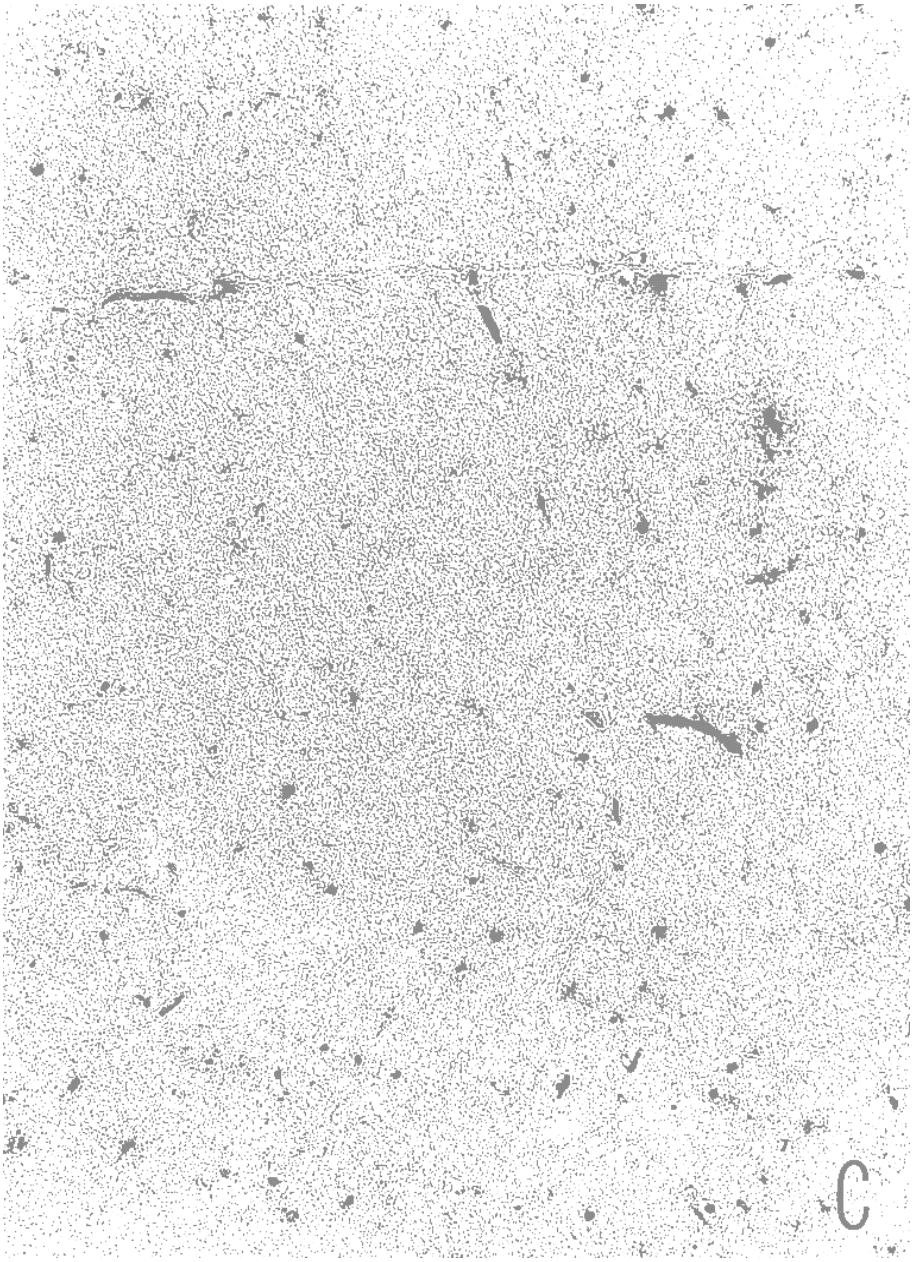

Fig. 4. - continued

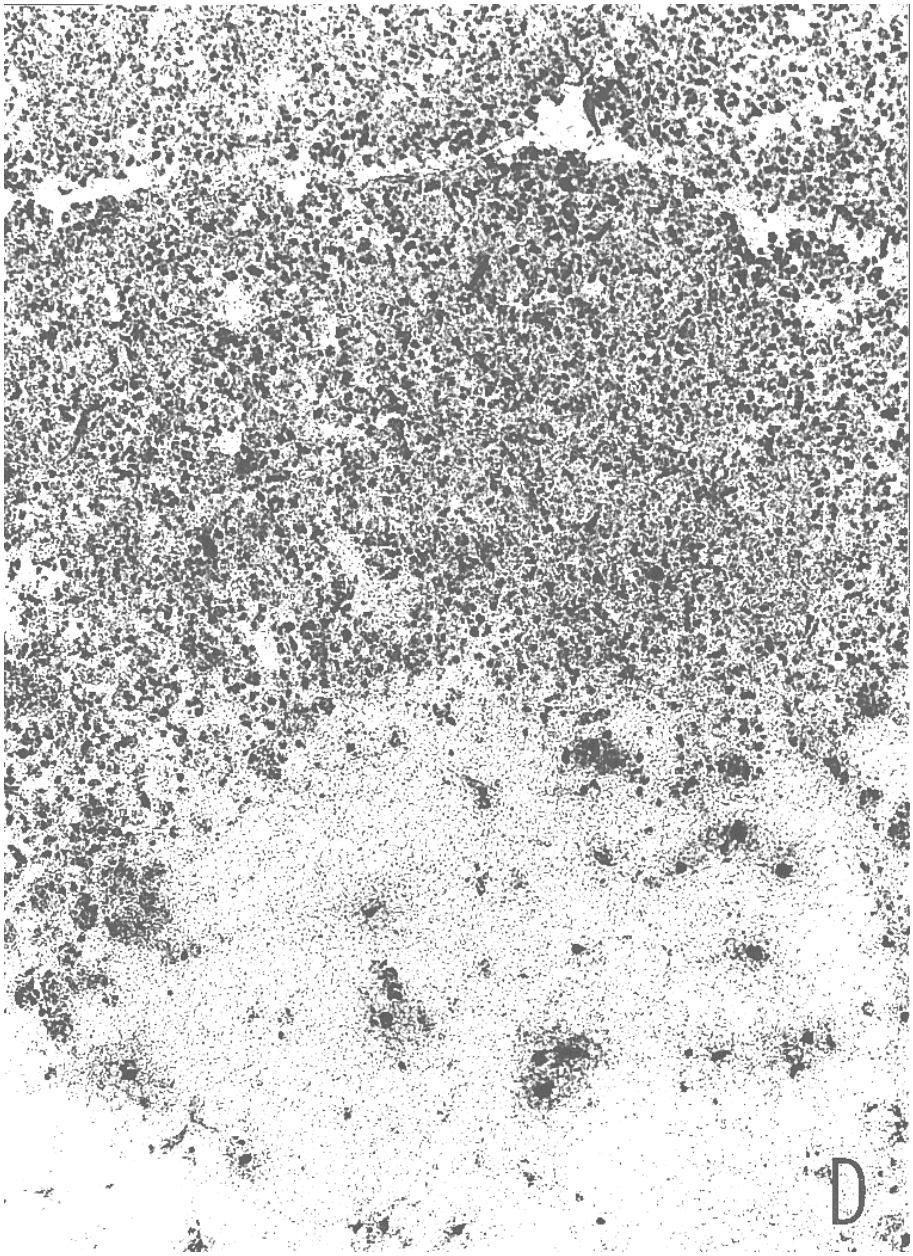

Fig. 4. - continued

Table 1. Number of total and large thymocytes per thymus on days 1 and 2 after dosing.

	DAY 1		DAY 2	
Compound	**Total**	**Large**	**Total**	**Large**
Control	70.8 ± 17.9	3.7 ± 1.0	102.5 ± 14.1	5.0 ± 0.6
DEX	19.8 ± 4.6*	0.7 ± 0.1*	9.2 ± 10.6*	0.6 ± 0.5*
TBTC	71.6 ± 17.8	2.8 ± 0.7	59.2 ± 22.3**	1.6 ± 0.9*
DBTC	87.0 ± 9.4	3.0 ± 0.2	61.5 ± 25.1**	1.3 ± 0.6*

Note. cell numbers ($x10^7$) are given as mean ± S.D., n=4.
* $p < 0.05$, compared to groups not indicated. ** $p < 0.05$, organotins compared to control.

DISCUSSION

In the present study it is shown that both DBTC and TBTC induce DNA fragmentation in freshly isolated rat thymocytes as well as splenocytes. *In vivo* data, however, demonstrate that in contrast to the glucocorticosteroid DEX, neither of these compounds increases the incidence of apoptotic cells in the thymus after a single oral dose (20 mg/kg) that selectively inhibits proliferation of immature thymocytes at 24-48 hr after exposure (Snoeij *et al.*, 1988; Pieters *et al.*, 1992; Table 1).

In vitro, both organotin compounds significantly increase apoptosis after short-term (10 min) incubation at the relatively high concentration of 3 µM, whereas after continuous exposure for 22 hr an optimum in DNA fragmentation was observed at 1 µM TBTC or 0.3 µM DBTC. Apparently, like other *in vitro* effects of TBTC (Zucker *et al.,* 1992), induction of apoptosis by TBTC or DBTC depends on both concentration and time of incubation. Thus, at higher concentrations and after prolonged exposure organotins may induce necrotic instead of apoptotic cell death, resulting in decreased DNA fragmentation. This may also explain why others (Aw *et al.*, 1990; Raffray and Cohen, 1993) could not detect DNA fragmentation in thymocyte suspensions after 1-6 hr of incubation at concentrations over 0.3 µM DBTC.

Thymus atrophy induced by DEX was clearly distinguishable from the one induced by organotins. Notably, DEX caused a dramatic decrease in the number of both total and large thymocytes and induced a profound increase of DNA fragmentation on day 1 and less on day 2 after dosing. The fast kinetics and nonselective decrease in size-characterized subpopulations seem typical for glucocorticoid-induced thymus atrophy (Lundberg, 1991) and strengthen the idea that the thymotoxic effects of organotins at relevant doses are not caused by stress-induced elevation of glucocorticosteroids (Seinen and Willems, 1976; Snoeij *et al.,* 1988).

The absence of apoptosis 1 or 2 days after DBTC or TBTC treatment is evidenced by flow cytometry and by *in situ* staining of DNA strandbreaks, whereas a previous study using the *in situ* BrdU method revealed an almost complete absence of proliferating cells on day 2 (Pieters *et al*., 1993a,b). Our present *in vivo* findings with TBTC contradict a previous study showing an increase in the incidence of DNA fragmentation 48 hr after single oral dosing of 30 or 60 mg TBTC/kg (Raffray and Cohen, 1993). However, part of this relative increase in apoptosis may be explained by the organotin-induced phenotypical shift in thymocyte subsets on day 2, which is characterized by a decrease of immature proliferating $CD4^-CD8^+$ and $CD4^+CD8^+$ cells and a relative increase of small and apoptotic $CD4^+CD8^+$ thymocyte subset (Pieters *et al.,* 1992). As these immature proliferating cells are less prone to apoptosis (Scollay *et al*., 1988; Kendall, 1990; Cohen, 1991) this shift may cause a relative increase in apoptosis. In addition, although no increases in adrenal weights were observed (Raffray and Cohen, 1993) stress-induced thymotoxicity cannot be excluded at the relatively high doses of 30 and 60 mg TBTC/kg body wt, as previous studies indicate that single doses over 20-35 mg TBTC/kg also reduce spleen and body weights (Snoeij *et al.,* 1988).

Data together indicate that apoptosis may be involved in organotin-induced thymus atrophy at high doses, but it is not evident at lower doses that selectively inhibit immature thymocyte proliferation. It should be noted here that both organotin compounds very effectively inhibit proliferation of thymocytes *in vitro* at concentrations as low as 0.1 µM (Snoeij *et al.,* 1986; Pieters *et al*., 1994). Combined with the observation that these thymus-selective organotins also induce apoptosis in isolated spleen cells and do not

accumulate in the thymus (Penninks *et al.,* 1987), this also argues against the relevance of apoptosis in the initiation of thymus atrophy at antiproliferative doses.

In summary, we demonstrate that although both thymotoxic organotin compounds DBTC and TBTC induce apoptosis *in vitro* at relatively high concentrations, apoptosis is not likely to be involved in organotin-induced thymus atrophy at a dose that selectively inhibits immature thymocyte proliferation. Organotin compounds are nevertheless very useful in examining mechanisms of chemical-induced apoptosis *in vitro*, considering that both DBTC and TBTC have a typical mode of action in causing DNA fragmentation (McConkey *et al*., 1994), which is dissimilar and even antagonistic from the one induced by glucocorticosteroids or via the T cell receptor (Zucker *et al.,* 1994).

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Chapter three

ORGANOTIN-INDUCED APOPTOSIS OCCURS IN SMALL CD4$^+$CD8$^+$ THYMOCYTES AND IS ACCOMPANIED BY INCREASED RNA SYNTHESIS

ALESSANDRA GENNARI, MARIANNE BOL, WILLEM SEINEN, ANDRÉ PENNINKS* AND RAYMOND PIETERS

Research Institute of Toxicology, Immunotoxicology Section, Utrecht University, PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

*TNO-Nutrition and Food Research Institute, Zeist, The Netherlands

ABSTRACT

The organotin compounds di-*n*-butyltin dichloride (DBTC) and tri-*n*-butyltin chloride (TBTC) are known to induce thymus atrophy in rats, by interfering with immature thymocyte proliferation. Higher doses of tributyltin compounds also induce apoptotic cell death. *In vitro*, a similar concentration-dependency was observed, i.e. low concentrations of organotins inhibit DNA synthesis and higher concentrations induce apoptosis. The mechanism of apoptosis by organotins is not exactly known, but their capacity to effectively inhibit protein synthesis seems to contradict with the idea that macromolecular synthesis is required for apoptosis. Therefore, we aimed to evaluate the relation between apoptosis and the synthesis of RNA and proteins (including heat shock proteins), with a focus on the apoptosis-sensitive thymocyte subset and the most thymotoxic organotin compound DBTC. Results showed that DBTC increased RNA synthesis, while inhibiting protein and DNA synthesis. This increase in RNA synthesis occurred in small high-density $CD4^+CD8^+$ thymocytes, which comprised cells showing high incidence of DNA fragmentation. Moreover, co-exposure to the RNA synthesis inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide protected cells from apoptotic cell death by DBTC or TBTC. Finally, organotin compounds were found to induce synthesis of the heat shock protein HSC73. Heat shock treatment (42°C, 20 min) did not initiate apoptosis in rat thymocytes, neither antagonized organotin-induced DNA fragmentation. Results together indicate that organotin-induced apoptosis involves the synthesis of yet unidentified RNA-molecules and proteins, and additionally, that the subset of thymocytes that is subject to apoptosis by organotins, i.e. the $CD4^+CD8^+$ subset, differs from the one (immature blastoid $CD4^-CD8^+$ and $CD4^+CD8^+$) that was previously characterized as being sensitive to the anti-proliferative effects of organotins.

INTRODUCTION

Derivates of dialkyltin compounds, like di-*n*-butyltin dichloride (DBTC), are used in various industrial applications such as PVC-stabilizers and catalyzers. Trialkyltin compounds, like tri-*n*-butyltin chloride (TBTC), are used as biocides for instance in agriculture, in anti-fouling paints and in fabrics (Fent, 1996). The toxicity of these organotins towards the immune system is well known, with atrophy of the thymus cortex

as the most obvious effect of both DBTC and TBTC (Seinen and Willems, 1976; Seinen *et al*., 1977; Seinen *et al*., 1979; Krajnc *et al*., 1984; Snoeij *et al*., 1988). In a series of studies we have shown that relative low doses (15-20 mg/kg body weight) of these organotins selectively inhibit the proliferation of immature $CD4^{-}CD8^{+}CD3^{-/low}$ and $CD4^{+}CD8^{+}CD3^{-/low}$ thymoblasts without signs of apoptotic cell death (Pieters *et al*., 1992; Gennari *et al*., 1997, see also chapter 2). Others have shown that at higher doses of in particular TBTO (tributyltin oxide) apoptosis was the predominating process leading to thymocyte depletion (Aw *et al*., 1990; Raffray and Cohen, 1993a). A similar concentration-dependent effect was observed *in vitro*. Low concentrations of either one of these organotin compounds caused inhibition of DNA synthesis (Seinen *et al*., 1979; Snoeij *et al.,* 1986), whereas at higher concentrations these chemicals induced cell death by apoptosis (Aw *et al*., 1990; Raffray and Cohen, 1993a). At even higher concentrations necrosis was the prevailing mode of cell death (Raffray *et al*., 1993b; Gennari *et al.,* 1997, see also chapter 2).

The process of apoptotic cell death by TBTC in particular, has been studied in some detail. It is shown that TBTC has a Ca^{2+} ionophoric effect that is claimed to trigger endonuclease-dependent DNA fragmentation without the need of macromolecular (protein and RNA) synthesis (Aw *et al*., 1990; Raffray *et al*., 1993b). Other studies however, demonstrated that TBTC at concentrations known to cause apoptosis, also stimulated RNA synthesis (Snoeij *et al*., 1986) and *de novo* synthesis of heat shock proteins (HSPs) (Zhang and Liu, 1992). For DBTC no mechanistic information exists regarding apoptosis, mainly because the capacity of this compound to induce apoptosis was unnoticed until recently when this effect was reported to be time-dependent (Gennari *et al*., 1997, see also chapter 2). Briefly, apoptosis by DBTC could not be detected after 1 h of incubation (Aw *et al*., 1990; Raffray and Cohen, 1993a), but after short-term incubation followed by overnight culture or continuous exposure for 22 h DNA fragmentation could be readily detected (Gennari *et al*., 1997, see also chapter 2).

The present study was performed to investigate the relation between organotin-induced apoptosis and synthesis of RNA and proteins, in particular for DBTC since this compound is regarded as the most effective thymotoxic organotin compound and presumably the active metabolite of TBTC (Snoeij *et al*., 1988).

MATERIALS AND METHODS

Chemicals and solutions

DBTC (Johnson Matthay Alfa Products, Karlsuhe, Germany), TBTC (gift from Dr. H. Meinema, TNO, Zeist, The Netherlands), DEX (10 µM, Sigma, St. Louis, MO, USA) and actinomycin D (Act D, 0.16 µM, Sigma) were dissolved in ethanol absolute (final ethanol concentration 0.1%, which is ineffective by itself) and then diluted in RPMI. Cycloheximide (CHX, 10 µM, Sigma) was dissolved in bidest. Propidium Iodide (PI, Sigma) was dissolved in PBS to obtain a concentration of 5 µg/ml. Ribonuclease A (RNase, type I-A, Sigma) was dissolved first in TRIS-buffer (10 mg/ml), boiled for 15 min, and then diluted in RNase buffer (0.1% sodium-citrate, 0.1% triton X-100) to obtain a lysis buffer with a final concentration of 0.5 mg/ml RNase. RPMI-1640 (Gibco, Paisley, U.K.) was used supplemented with 5% heat-inactivated fetal calf serum (Gibco), 1% glutamine (Gibco), penicillin/streptomycin (Flow, Irvine, U.K.), sodium bicarbonate (Gibco), 1% HEPES buffer (Gibco). PBS was supplemented with 4 mM Ca^{2+}, 10 mM Mg^{2+} and 2 mM glucose.

Tran ^{35}S-label (ICN Biomedicals Inc., Irvin, USA), Hyperfilm B-max (Amersham, `s Hertogenbosch, The Netherlands) and Immobilon-P membranes (Immobilon PVDF Transfer, Millipore, Etten-Leur, The Netherlands), were used for metabolic labeling experiments. Blots were stained with specific monoclonal antibody directed to HSP72 (clone N27F3-4, Stressgen, Victoria, Canada) and HSC73/HSP72 (clone C92F3A-5, Stressgen). ^{3}H-Methyl-Thymidine (^{3}H-TdR), ^{3}H-Uridine (^{3}H-Urd), ^{14}C-Leucine (^{14}C-Leu) and ^{35}S-tran (containing ^{35}S-methionine) were purchased from the Radiochemical Center, Amersham, UK.

OX8 (anti-CD8)- and ER2 (anti-CD4)-producing hybridomas were cultured in-house (OX-8 line obtained from European Collection of Animal Cell Cultures, Salisbury, U.K. and ER2 from Dr. J. Rozing, University of Groningen, The Netherlands). Biotine conjugated OX8 and ER2-FITC were used to detect CD4 vs. CD8 positivity of thymocyte subsets. Streptavidin-phycoerythrine (Southern Biotechnology Association, SBA, Inc., Birmingham, USA) was used to visualize binding of OX8-biotine.

Animals

Male rats, used at the age of 4-5 weeks, were bred and housed at the Animal Facilities of the Utrecht University. They were kept in filter-top macrolon cages with *ad libitum* food and acidified drinking water. Rats were killed by decapitation and thymi were aseptically removed without adjacent parathymic lymph nodes.

Cell suspensions and culture

Thymocyte suspensions were prepared by crushing the thymus on a nylon sieve and collecting the cells in ice-cold PBS. After centrifugation, cells were resuspended in RPMI 1640-supplemented, incubated for 10 min in a water bath (37°C) with the compounds, washed twice, and then incubated overnight (22 h) at 37°C without compounds. DEX, Act D and CHX were present throughout the whole incubation period of 22 h.

Centrifugal elutriation

Centrifugal elutriation of thymocytes was performed as described by Snoeij *et al.* (1986), using a Beckman J2-21 centrifuge equipped with a JE-6B elutriator rotor. The flow was generated by hydrostatic pressure. An amount of 3-5x10^8 cells was introduced with a flow of 12 ml/min and a rotor speed of 3200 rpm. After 5 min of equilibration, the first fraction was obtained by increasing the flow to 18 ml/min. Then, 6 additional fractions were collected by stepwise decreasing the rotor speed to 3000, 2900, 2800, 2600, 2200 and 2000 rpm. All fractions were obtained in volumes of 50 ml. After sedimentation at 200 g for 10 min, fractions collected at 3200, 3000 and 2900 rpm were pooled as small-sized cells, at 2800 and 2600 rpm as middle-sized cells and at 2200 and 2000 rpm as large-sized cells.

DNA, RNA and protein synthesis detection

The effect of DBTC on DNA, RNA and protein synthesis was estimated by determination of the incorporation rate of ^{3}H-TdR, ^{3}H-Urd, ^{14}C-Leu into acid-precipitable material. After a 30 min pre-incubation period with graded concentrations of organotin compounds, 1 µCi/ml ^{3}H-TdR (final concentration 20 nM), 1 µCi/ml ^{3}H-Urd (final concentration 20 nM) or 50 nCi/ml ^{14}C-Leu (final concentration 145 µM) was added to

rat thymocyte suspensions ($2x10^7$ cells/ml). At regular intervals up to 60 min after addition of the label, samples were taken in 4-fold and cells were harvested onto glass fiber filters using a 5%-solution of trichloroacetic acid. Radioactivity was counted in a Kontron MR 300 liquid scintillation counter.

Induction of heat shock response

Cells resuspended in RPMI 1640-supplemented, were incubated at 42℃ for 20 min in a water bath and then incubated at 37℃ overnight. In case of cells treated with compounds, after heat shock induction, thymocytes were incubated 10 min with DBTC or TBTC, washed twice and then incubated at 37℃ overnight without compounds.

HSP detection

Suspensions of thymocytes were prepared as described above, and cells were exposed to graded concentrations of the test compounds in 96 well plates, 10^6 cells per well in RPMI without methionine or serum. Following pre-incubation with chemicals for 30 min at 37℃, ^{35}S-tran was added to a final activity of 25 µCi/ml and cells were labeled for 2 h in the continuous presence of compounds. Control and heat-shock samples were incubated in test vials. After labeling, cells were washed three times in ice-cold phosphate-buffered saline (PBS), and lysed in 50 µl Laemmli sample buffer. Aliquots of the samples were harvested on glassfiber filters and incorporated radioactivity was measured to determine total protein synthesis.

Samples were then run on 10% SDS-polyacrylamide slab gels in a discontinuous buffer system. Separated proteins were blotted onto a membrane that was used for autoradiography and/or immunoblotting. Autoradiograms were obtained from radiolabeled proteins on the blots by direct exposure of films at room temperature for 2-7 days. For further characterization of heat shock proteins, membranes were blocked with Tris-buffered saline/1% milk powder for 1 h and then incubated with a mixture of the two anti-HSP antibodies for another hour. Thereafter, membranes were washed three times with TBS/0.05% Tween 20 (TBS/T) and incubated with the appropriate dilutions of appropriate alkaline phosphate conjugated second step antibody for another hour (goat-anti-mouse-IgG-AP, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA;

anti-rat-IgG-AP, Sigma Chemical Company, St. Louis, MO, USA; anti-rabbit-IgG-AP, Boerhinger Mannheim GmbH Biochemica, Mannheim, Germany). After three more washes in TBS/T, bands were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in Tris buffer, pH 9.5 (20 min).

Detection of apoptosis

Detection of DNA fragmentation by flow cytometry with PI as fluorescent indicator was done essentially as described by Nicoletti *et al.* (1991). Briefly, after incubation with the test compounds, $5x10^6$ cells/ml were centrifuged and resuspended in 1 ml PBS; then 200 µl of this cell suspension was incubated with 0.5 ml RNase-containing lysis buffer for 30 min at room temperature. Thereafter, 0.5 ml PI (5 µg/ml) was added and the fluorescence of individual nuclei was measured (in the FL_2 channel set for log scale) using a FACScan flow cytometer (Becton-Dickinson, Erembodegem, Belgium).

A second method was used to characterize the thymocyte subset affected by organotin-mediated apoptosis. This protocol, described by Swat *et al.* (1991), uses forward scatter (FSC)/side scatter (SSC) and CD4/CD8 expression as characteristics for apoptotic cells (apoptotic thymocytes have a low FSC and a high SSC and low expressions of CD4 and CD8). Briefly, thymocytes were incubated in PBS/1% (w/v) BSA with optimal dilutions of OX8-biot and ER2-FITC for 30 min, washed and stained for 30 min with streptavidin-phycoerythrin. After a final washing, FSC/SSC and CD4/CD8 staining patterns were analysed on a FACScan flow cytometer (Becton Dickinson, Erembodegem, Belgium, equipped with a single argon laser).

Statistics

Statistical significance was determined by Student's t-test or Dunnett's multiple comparison test after analysis of variance.

RESULTS

DBTC increases the synthesis of RNA in small thymocytes

Previously, TBTC was shown to increase the synthesis of RNA in rat thymocytes. Here, we demonstrate that also DBTC is able to do so (fig. 1). After an incubation period of 30 min, RNA synthesis increased in a concentration-dependent manner up to an optimum at a concentration of 5 µM DBTC. At 10 µM RNA synthesis remained at control level. The inhibitory EC50s for DNA and constitutive protein synthesis for DBTC in these studies were of 3.0 and 0.6 µM, respectively (fig. 1).

Because it was known that DBTC had a profound anti-proliferative effect on large-sized thymocytes *in vivo* (Snoeij *et al*., 1988; Pieters *et al*., 1992; 1994), we next studied whether the effects of this organotin compound on the synthesis of RNA were confined to a same subset of thymocytes. Fig. 2 illustrates however, that the smallest cells (diameter between 4.3 µM and 6.8 µM) show a dose-dependent increase in the synthesis of RNA, reaching 447% of control level at 5 µM of DBTC. RNA synthesis was unaffected in middle-sized (6.8 µM to 8.6 µM) and large-sized thymocytes (over 8.6 µM).

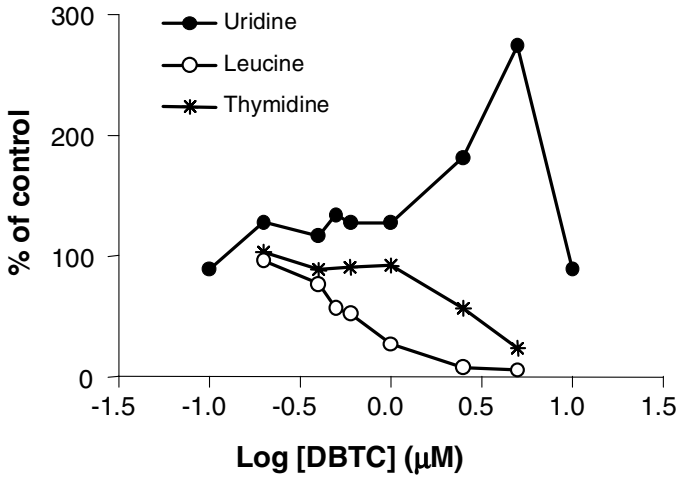

Fig. 1. Effects of DBTC on ^{3}H-Urd, ^{3}H-TdR and ^{14}C-Leu incorporation in rat thymocytes, after an incubation period of 30 min. The organotin compound was used at concentrations from 0.1 µM up to 10 µM. The maximum increase of RNA synthesis is obtained at 5 µM DBTC, whereas the half-maximal inhibition for DNA and protein synthesis occurs at 3 and 0.6 µM DBTC, respectively. Values represent one typical experiment.

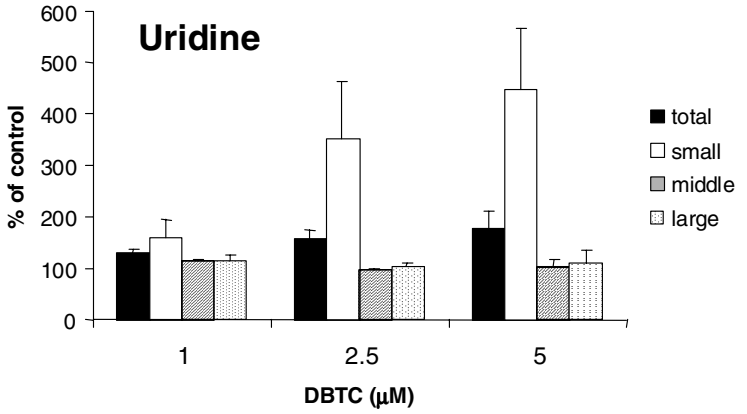

Fig. 2. Effects of DBTC on ^{3}H-Uridine incorporation on subsets of thymocytes: small (diameter between 4.3 µM and 6.8 µM), middle (diameter between 6.8 µM and 8.6 µM) and large cells (diameter above 8.6 µM). Increase of uridine incorporation is DBTC-concentration-dependent. Values are means ± SD of three separate experiments.

Organotin-induced apoptosis occurs in the $CD4^{+}CD8^{+}$ subset

It is well known that the majority of small thymocytes belongs to the $CD4^{+}CD8^{+}$ subset. Therefore, we used the method of Swat *et al.* (1991) to detect apoptosis in thymocytes as this method enables characterization of apoptosis in $CD4^{+}CD8^{+}$ thymocytes. We observed that after 10 min exposure, followed by overnight culture without compounds, both DBTC (1 µM and 5 µM, fig. 3) and TBTC (not shown) caused a shift of normal viable thymocytes (normal FSC, and normal or low SSC, corresponding with $CD4^{high}CD8^{high}$ thymocytes) to cells with a lower FSC and a higher SSC (corresponding with $CD4^{low}CD8^{low}$ thymocytes). The simultaneous shift of the percentage of $CD4^{high}CD8^{high}$ thymocytes in favour of $CD4^{low}CD8^{low}$ thymocytes indicates that organotin-induced apoptosis takes place in $CD4^{+}CD8^{+}$ thymocytes.

Inhibition of RNA and protein synthesis decreases organotin-induced apoptosis

From the above results we argued that the increased synthesis of RNA was linked to the induction of apoptosis. Therefore, we incubated rat thymocytes with combinations of organotins and the classical RNA synthesis inhibitor Act D. In addition, because apoptosis in many cases is protein synthesis-dependent, we also examined whether the protein synthesis inhibitor CHX was able to inhibit organotin-induced apoptosis. In fig. 4

it is shown that both inhibitors are able to decrease the fragmentation of DNA induced by DBTC (1 µM) or TBTC (1 µM) after 10 min exposure, followed by overnight culture without organotins but still in presence of the inhibitors. For Act D however, the decrease of apoptosis appeared more effective in case of DBTC (27%) than in case of TBTC (15%), whereas CHX inhibited TBTC- and DBTC-induced apoptosis to the same extent. These inhibitors by themselves did not induce DNA fragmentation and, like expected, they inhibited DEX-induced apoptosis.

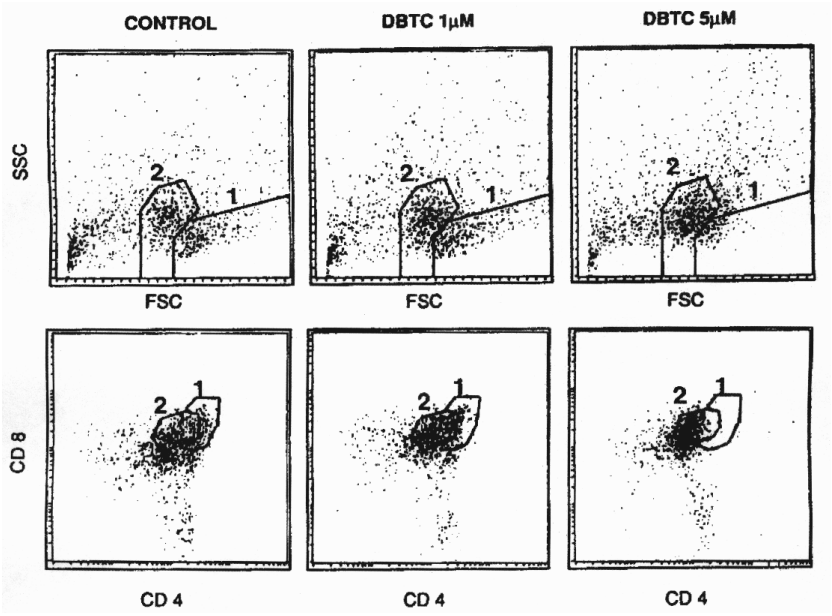

Fig. 3. Induction of apoptosis by DBTC (10 min, then overnight culture without compound) in thymocytes, analysed by FACS. Upper panels show cell-size (FSC) vs. cell density (SSC) and lower panels show CD4 vs. CD8 staining. Gate 1 is set for viable thymocytes (normal FSC and SSC) and corresponds with $CD4^{high}CD8^{high}$ thymocytes and gate 2 is set for $SSC^{high}FSC^{low}$ cells, corresponding to apoptotic $CD4^{low}CD8^{low}$ thymocytes.

DBTC and TBTC stimulate synthesis of the heat shock protein HSC73

Because the organotins used in this study are effective inhibitors of protein synthesis by themselves, the protective effect of CHX was somewhat surprising. Apparently, some proteins are still synthesized or even up-regulated under the influence of organotins. Analyses of *de novo* protein synthesis in the presence of DBTC or TBTC revealed the

appearance of HSC73 (fig 5A). Other HSPs, as observed in case of heat shock treatment, were not detected. The classical protein inhibitor CHX was also found to induce proteins between 38 and 56 KDa, but not HSC73 (fig 5B). Although we used ^{35}S-methionin as protein precursor in this series of experiments, the EC50 value was the same as with ^{14}C-Leu as precursor (data not shown), and importantly, in all cases HSP was detected only when the inhibition of the protein synthesis was over 20%.

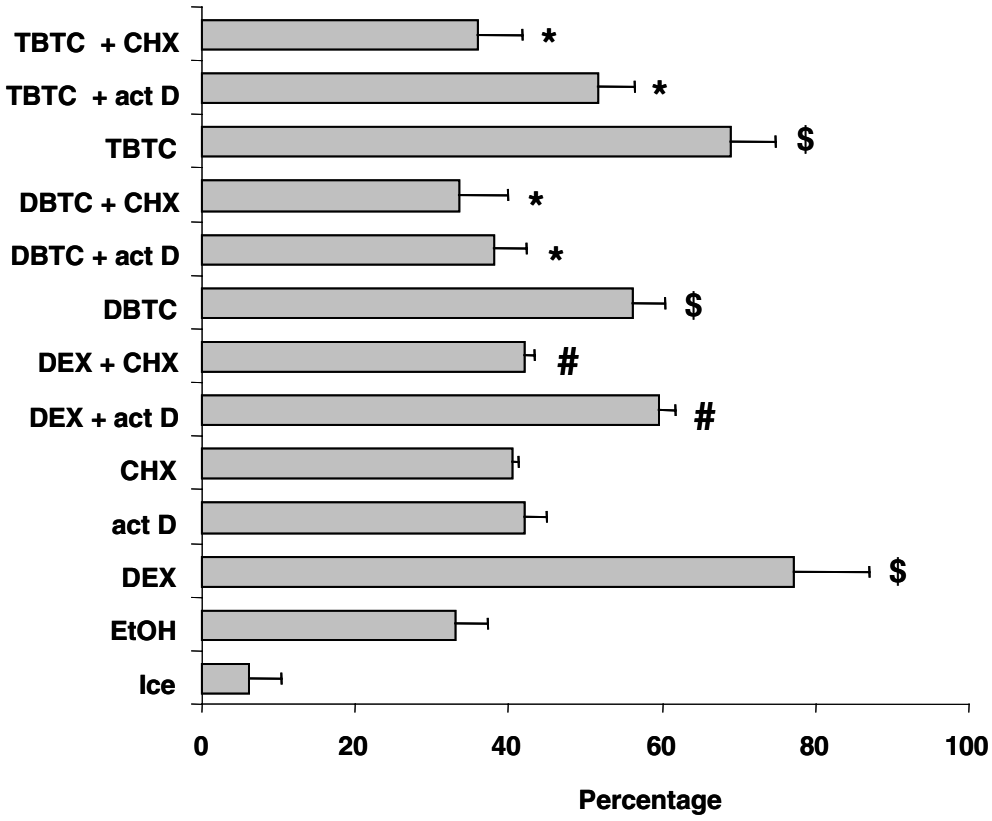

Fig. 4. DNA fragmentation measured by propidium iodide method on flowcytometer in thymocytes treated with DBTC (1µM), TBTC (1 µM) or DEX (10 µM) in combination with the RNA synthesis inhibitor Act D (0.16 µM) and the protein synthesis inhibitor CHX (10 µM). Values are means ± SD of three separate experiments. $ p<0.05, compared to EtOH (negative control), # p<0.05, compared to DEX (positive control), * p<0.05, compared to organotin compounds.

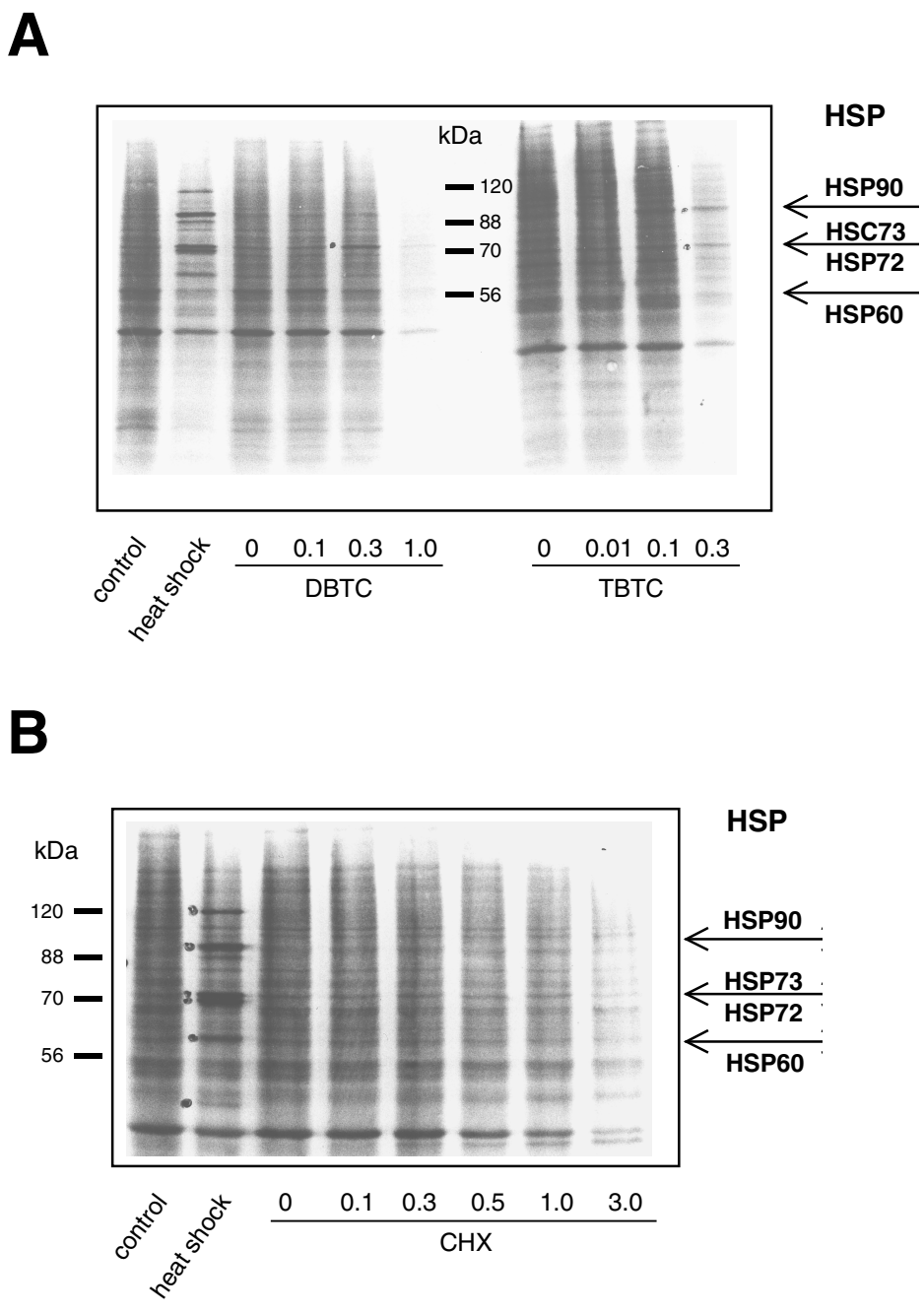

Fig. 5. SDS-polyacrylamide gel electrophoresis of proteins extracted from thymocytes incubated with increasing concentrations of DBTC or TBTC (from 0 to 1µM) (A) or with the protein synthesis inhibitor CHX (B). Cells treated for 2.5 hrs with organotin compounds showed induction of HSC73, whereas CHX induced synthesis of various proteins in the range of 38-56 KDa, but not of HSC73.

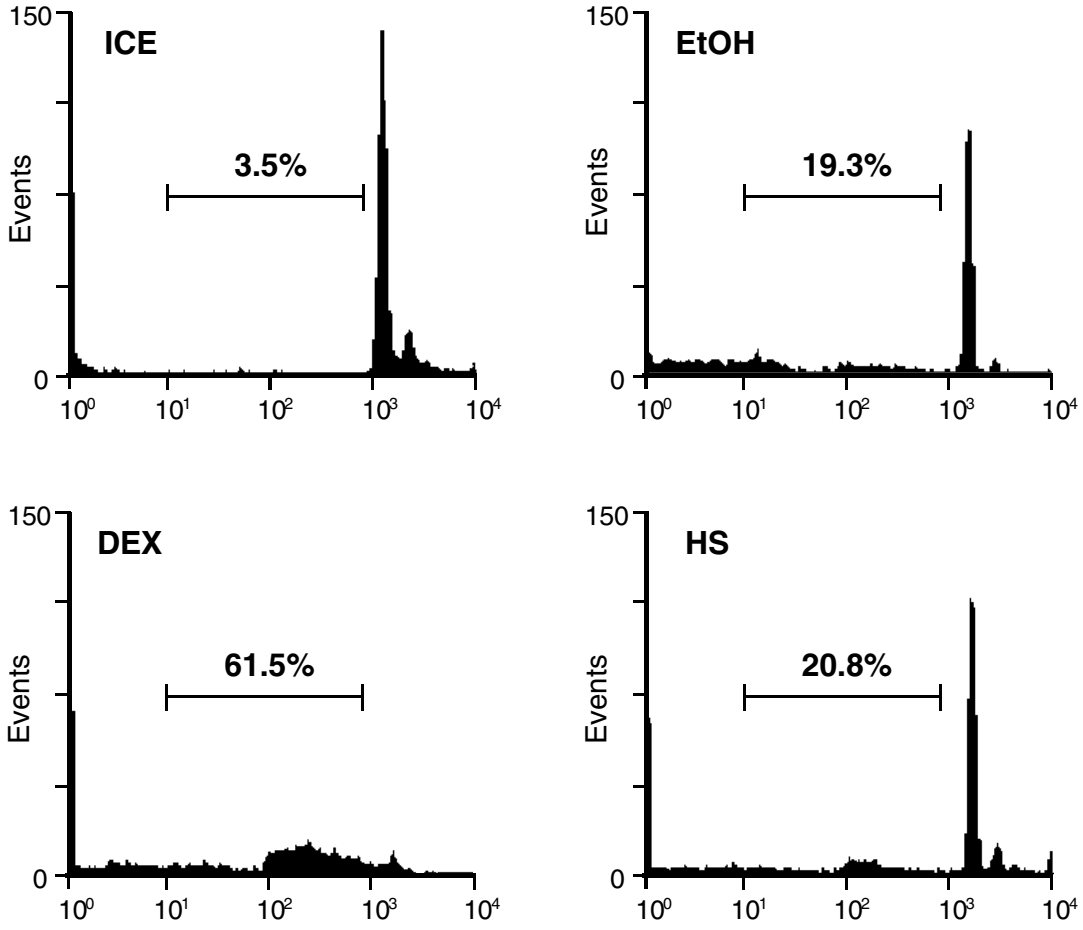

Fig. 6. Quantitative analysis of apoptosis by flow cytometry in thymocytes treated with EtOH (negative control), DEX (10 µM, positive control) or HS (42℃ for 20 min). The amount of apoptotic nuclei is indicated as percentage. Values are means ± SD of three separate experiments.

Heat shock does not antagonize initiation of apoptosis induced by organotins

Others (Migliorati *et al*., 1992) have shown that HSPs induced by heat shock (42°C, 20 min) are able to protect mouse thymocytes from glucocorticosteroid-induced apoptosis, whereas on the other hand HSPs by themselves initiate apoptosis. However, in rat thymocytes, heat shock treatment appeared unable to induce apoptosis (fig. 6) and in addition did not protect rat thymocytes from organotin- nor glucocorticosteroid (dexamethasone)-induced apoptosis (fig. 7).

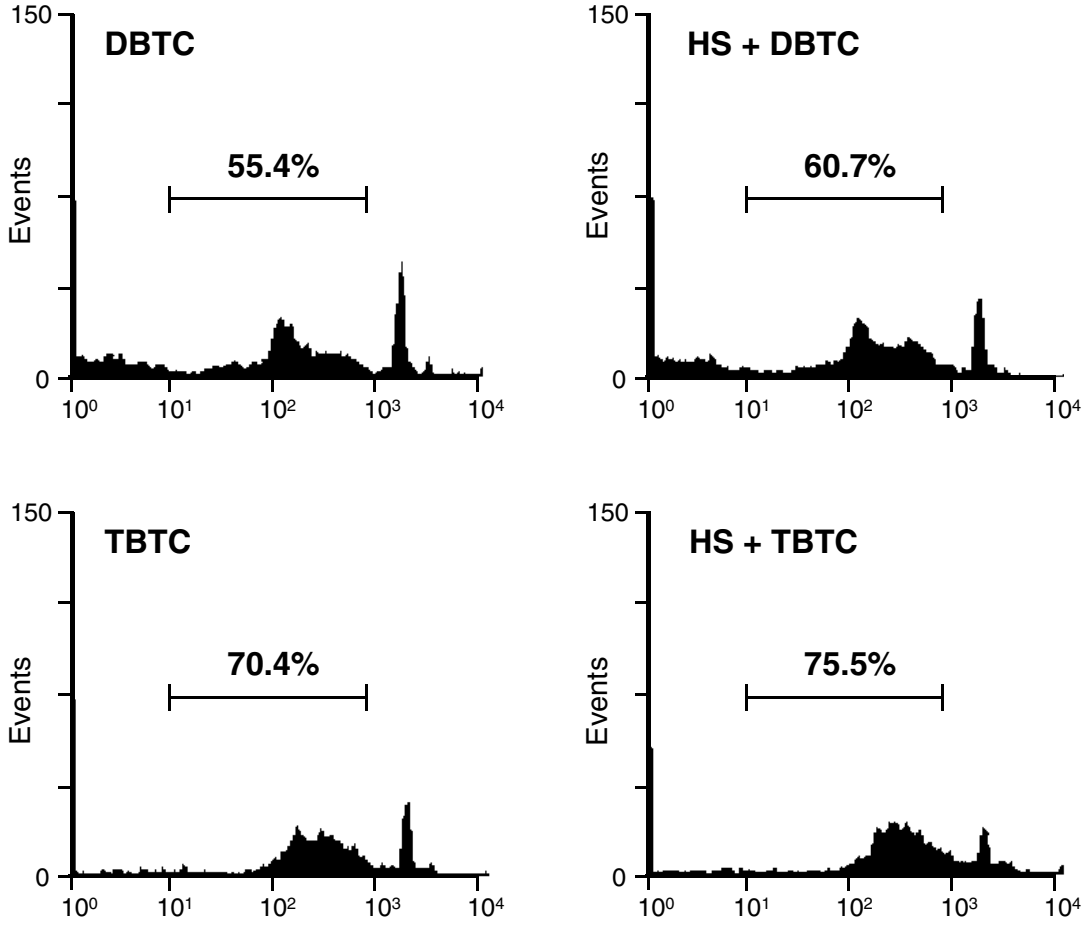

Fig. 7. Flow cytometry of PI-stained nuclei of thymocytes treated with HS (42℃ for 20 min) alone or with HS in combination with 1 μM DBTC or TBTC. Values are means ± SD of three separate experiments.

DISCUSSION

Results described in this chapter show that the thymotoxic organotin compound DBTC increases the synthesis of RNA in a concentration-dependent manner, but most clearly at relative high concentrations (an optimum of synthesis is obtained at 5 μM DBTC). Like demonstrated before in case of TBTC (Snoeij *et al*., 1986), the increase of RNA occurs in small-sized, high-density thymocytes. Previously, we have found that both TBTC and DBTC were able to cause apoptosis in rat thymocytes in a time- and concentration-dependent manner (Snoeij *et al*., 1988). Now, we show that organotin-induced apoptosis seems to affect $CD4^+CD8^+$ thymocytes in particular, and that the process of DNA

fragmentation is RNA- and protein-synthesis-dependent. It is worthy to note that thymocyte selection takes place in small $CD4^+CD8^+$ thymocytes and that they are vulnerable to initiators of apoptosis (Swat *et al*., 1991). Apparently, organotin compounds enhance apoptosis *in vitro* in a subset of thymocytes that is different from the blastoid and proliferating immature $CD4^-CD8^+TcR\alpha\beta^{-/low}$ or $CD4^+CD8^+TcR\alpha\beta^{-/low}$ subsets. The latter two subsets are suggested to be the primary intrathymic targets *in vivo* in case of relatively low doses of organotins (Pieters *et al*., 1992; 1994). This does not exclude, however, the possibility that apoptosis occurs in these $CD4^+CD8^+$ thymocytes when higher doses of organotins are used (Gennari *et al*., 1997, see also chapter 2).

The present data on the importance of protein are in contradiction with previous data by Raffray *et al*. (1993b). They showed that pre-incubation with CHX had no effect on the induction of apoptosis by tributyltin oxide (TBTO). However, the observed inhibitory effects in the present study occur during overnight incubation of the cells with CHX, whereas these previous studies used short-term (10 min) pre-exposures with the inhibitors. Together, the role of *de novo* synthesis of proteins remains unclear, certainly considering the potent protein inhibiting effects of these thymotoxic organotins.

Both organotin compounds initiate HSC73 synthesis at concentrations that inhibit protein synthesis to around 20% of control. Previously, Migliorati *et al.* (1992), showed that heat shock (HS) was able to induce apoptosis in mouse thymocytes and moreover to protect cells from glucocorticoid-induced cell death. Present data show that HS in rat thymocytes is not capable to significantly increase DNA fragmentation, or to inhibit organotin- or DEX-mediated apoptosis. The observed difference may be due to species-specifity.

Because CHX, which does not induce apoptosis in thymocytes, also induces synthesis of HSPs, although a different pattern, it can be argued that induction of HSC73 by DBTC or TBTC is not due to a specific, direct effect of organotin compounds, but rather a consequence of suppression of protein synthesis (Albers *et al*., 1996). So, although HSPs are not likely to be involved in the initiation phase of apoptosis, some (other) newly synthesized but yet undefined proteins seem to be required in particular possibly at late steps of organotin-induced apoptosis.

Future studies will address the characteristics of the proteins and the RNA molecules that are produced. This would enable mechanistic linkage between the present effects with the known capacity of the organotins to affect a variety of cellular and biochemical processes, including interference with ATP level (Snoeij *et al*., 1986; Marinovich *et al*., 1990), increase of Ca^{2+} influx (Chow *et al*., 1992), interaction with T cell receptor signalling (Pieters *et al*., 1995), cytoskeleton-activity (Chow *et al*., 1994), and mitochondrial activity (Corsini *et al*., 1997).

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Chapter four

DBTC INDUCES THE SYNTHESIS OF *NUR77* AND GST INVOLVED IN THE APOPTOTIC PATHWAY

ALESSANDRA GENNARI[1,2], CORRADO LODOVICO GALLI[1], MARINA MARINOVICH[1], RAYMOND PIETERS[2], AND EMANUELA CORSINI[1]

[1]Laboratory of Toxicology, Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

[2]Research Institute of Toxicology, Immunotoxicology Section, Utrecht University, PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

ABSTRACT

The thymotoxic organotin compounds, as di-*n*-butyltin dichloride (DBTC) and tri-*n*-butytin chloride (TBTC) are known to induce apoptosis *in vitro* in rat thymocytes. They also affect macromolecular synthesis, inhibiting DNA synthesis and increasing RNA synthesis. Since these RNA molecules, likely to be involved in the initiation of the apoptotic process, have not been identified yet, the purpose of this research was to characterize, by a cDNA macro array, the expression of genes involved in apoptosis.

Results showed that two genes, namely glutathione-S transferase (GST), and *nur77* were rapidly transcripted following exposure of rat thymocytes to 3 μM DBTC. The products of both genes are known to be involved in the apoptotic process: GST is a defense enzyme aimed to cellular detoxification, while *nur77* is a transcription factor expressed in response to TCR mediated apoptosis in immature T cells.

Antisense oligonucleotide inhibition of *nur77* expression prevented apoptosis induced by DBTC, supporting a role of *nur77* in organotin-induced apoptotic cell death.

INTRODUCTION

Di- and tri-substituted organotin compounds are used in a variety of technical applications. Dialkyltin compounds, such as di-*n*-butyltin dichloride (DBTC) are used primarily as stabilizers for polyvinylchloride plastics, while the trisubstituted organotins, such as tri-*n*-butyltinchloride (TBTC), are widely used as biocides for the preservation of paper and wood, for disinfection of surfaces and cooling water, and as active ingredient in antifouling paints (Van der Kerk, 1978; Fent, 1996).

Both DBTC and TBTC are known to induce a selective, dose-dependent reduction of thymus weight and thymocyte number in rats (Seinen and Willems, 1976; Seinen *et al.,* 1977). *In vitro*, and possibly also *in vivo* at high doses, they induce apoptosis in rat thymocytes (Aw *et al.,* 1990; Raffray and Cohen, 1991; Gennari *et al.*, 1997, see also chapter 2). Dialkyltin and trialkyltin compounds are also potent metabolic inhibitors, and strongly reduce oxidative phosphorylation processes and ATP synthesis in mitochondria (Penninks *et al.*, 1983; Snoeij *et al.,* 1987). Mitochondria have also an important role in the pathway leading the cell to DNA fragmentation, being a source of reactive oxygen species (ROS) and of pro-apoptotic factors such as cytochrome *c* (McConkey *et al.*, 1989; Stridh *et*

al., 1998; Gennari *et al.*, 2000b, see also chapter 5). Furthermore, DBTC and TBTC are known to affect macromolecular synthesis, suppressing DNA and protein synthesis and increasing RNA synthesis (Snoeij *et al.*, 1986; Gennari *et al*., 2000a, see also chapter 3). In freshly isolated rat thymocytes, the increase of RNA synthesis occurred in the small high-density $CD4^+CD8^+$ subset, which comprised also of the cells sensitive to apoptosis after organotin compounds exposure (Snoeij *et al*., 1987; Gennari *et al.*, 2000a, see also chapter 3). Recent data showed that DNA fragmentation induced by organotins was RNA- and protein-synthesis-dependent (Gennari *et al*., 2000a, see also chapter 3), which is in agreement with the general idea that macromolecular synthesis is required for apoptosis. It has been shown that organotins initiated the synthesis of the heat shock protein HSC73 at concentrations that inhibited protein synthesis to around 20% of control. This induction of HSC73, however, appeared not to be a specific, direct effect of organotin compounds, but rather a consequence of suppression of protein synthesis (Gennari *et al.*, 2000a, see also chapter 3). So, other yet unknown neo-synthesized proteins seem to be required in organotin-induced DNA fragmentation. The aim of this study was to identify newly synthesized apoptosis-related RNA molecules induced by DBTC, and to characterize their involvement in the apoptotic process.

For this purpose, we used a commercially available rat cDNA macro array that allows the analysis of multiple genes simultaneously.

MATERIALS AND METHODS

Chemicals and solutions

Dibutyltin (DBTC) was obtained from Johnson Matthay Alfa Products (Karlsruhe, Germany) and propidium iodide (5 µg/ml PI) from Sigma (St. Louis, MO). Ribonuclease A (RNase, type I-A, Sigma) was dissolved first in Tris buffer (10 mg/ml) and boiled for 15 min and then diluted in RNase buffer (0.1% sodium citrate, 0.1% Triton X-100) to obtain a final concentration of 0.5 mg/ml. RPMI 1640 (Sigma) was supplemented with 5% heat-inactivated fetal calf serum (Sigma), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Animals

Four-five week old female Wistar rats (Charles River, Calco, Italy) were used for the experiments. Animals were housed three to four per cage on woodchip bedding, and allowed food and water *ad libitum*. Rats were killed with CO_2 and thymi were isolated.

Cell suspensions and incubation

Cell suspensions from thymi were prepared as described by Gennari *et al.* (1997, see also chapter 2). For the experiments, thymocytes were incubated with 3µM DBTC dissolved in ethanol absolute (EtOH) or with EtOH as vehicle control (the final EtOH concentration was 0.1%, ineffective by itself).

Apoptosis rat cDNA expression-array

The identification of cDNAs has been performed using the ″apoptosis rat cDNA expression macro-array kit″ (Clontech, Palo Alto, CA). Briefly, after 30 min of incubation with 0 or 3 µM DBTC, RNA was extracted from thymocytes as described by Chomczynski and Sacchi (1987) and 250 µg of total RNA was treated with DNase. After assessing yield and purity, 2 µg of RNA were used to be converted into α-^{32}P-labeled first strand cDNA and then hybridized to the Atlas Array membranes following instructions. After exposure of the membrane to x-ray film (Kodak X-OMAT) at -80°C dark spots, representing cDNAs, were identified following Clontech template.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed as previously described (Corsini *et al*., 1996) with slight modifications, in order to confirm the induced genes. Briefly, 2 µg of total RNA was resuspended in a 11.5 µl final volume of deionized H_2O and then 1 µl of oligod(T) (Perkin Elmer, Norwalk, CT) was added to each sample. After the samples reached 70°C for 10 min, the reaction mixture [0.1 M dithiothreitol (Gibco, Grand Island, NY), 10 mM dNTPmix (Perkin Elmer), First Strand Buffer (Gibco), Superscript reverse transcriptase (Gibco)] was added and tubes were incubated for 30 min at 42°C and for 15 min at 70°C. PCR primers for rat *nur77*, glutathione S-transferase (GST) and glyceraldehyde 3-phosphate dehydrogenase (GPDH, housekeeping gene) were synthesized by Primm (Milan,

Italy) and contained the following sequences: rat *nur77* sense: 5'-TTCCACCGAACTGC-AGCATC-3'; antisense: 5'-TACCCGAAGGGCCCGGACGT-3'; rat GST sense: 5'-TCG-CTCGGAACTTC GGTCTG-3'; antisense 5'-ACCAGGTGGTTCAGGACCGG-3'; mGPDH sense: 5'-CATGTAGGCCATGAGGTCCACCAC-3'; antisense 5'-TGAAGGT-CGGTGTGAACGGATTTGGC-3'. Five-microliter aliquots of the synthesized cDNA (200 ng RNA) were added to a mixture containing 2.5 µl PCR buffer (Perkin Elmer), 2 µl dNTPmix, 0.25 µl of specific primers, 0.25 µl Taq (Perkin Elmer), 1.88 µl $MgCl_2$. Amplifications were initiated with 6 min of denaturation at 95℃ for 1 cycle, followed by 35 cycles at 95℃ for 1 min, 57℃ for 1 min, and 72℃ for 1 min. After the last cycle of amplification, the samples were incubated for 10 min at 72℃.

The PCR products were visualized by UV illumination following electrophoresis through 2% agarose at 80 V for 1 h and staining in Tris-borate-EDTA (TBE) containing 0.5 µg/ml ethidium bromide. The amplified PCR products from total RNA were 478 bp for *nur77*, 389 bp for GST and 983 bp for GPDH. Gels were photographed with type 667 film (Polaroid, Cambridge, MA).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared essentially as described by Schreiber *et al*. (1989). After 10 min treatment with 0 or 3 µM DBTC, followed by 30 min incubation at 37℃ without compounds, rat thymocytes were washed once and resuspendend in 1 ml of a hypotonic lysis buffer (buffer A: 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Cells were incubated for 15 min, then 62.5 µl of a 10% Nonidet P-40 (Sigma) solution was added, and after 30 seconds of mixing they were centrifuged. The pelleted nuclei were washed once with 200 µl of buffer A plus 12.5 µl of 10% Nonidet P-40, centrifuged, suspended in 50 µl buffer C (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), mixed for 20 min, and then centrifuged. The supernatant containing nuclear proteins was harvested and protein concentration was determined (Bio-Rad Protein Assay). Electrophoretic mobility shift was performed as follows. Binding reaction mixtures (16 µl) containing 4 µg of nuclear extract, 2 µg poly dI-dC, 10.000 cpm ^{32}-P-labeled probe, 8 µl buffer D (10 mM Hepes, 2% Ficoll, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.4 µg/ml

albumin, 2 mM DTT) were incubated for 30 min at room temperature before separation in a 5% acrylamide gel in TBE followed by autoradiography. A double-strand oligonucleotide containing the binding site for *nur77* (5'-GGAGTTTTAAAAGGTCATGCTCAATTT-3', Primm) was labeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (Amersham).

Antisense oligonucleotide treatment and detection of apoptosis

The sense (5'-ATGGGTCCTCGCCTCAAGGAG-3') and antisense (5'-CTCCTTGAGGCGAGGACCCAT-3') phosphorothioate analogues of the oligonucleotides to the 5'-end of *nur77* mRNA were synthesized and obtained from Primm. Cells were preincubated for 30 min with oligonucleotides, followed by 10 min DBTC exposure and then overnight incubation with oligonucleotides without DBTC.

Detection of apoptosis by flow cytometry with PI as fluorescent indicator was done essentially as described by Nicoletti *et al.* (1991). Briefly, after incubation with the test compounds, $5x10^6$ cell/ml were centrifuged and resuspended in 1 ml PBS. Of this suspension 200 µl was incubated for 30 min with 0.5 ml RNase (0.5 mg/ml) at room temperature. Then, 0.5 ml PI was added (5 µg/ml, in PBS) and the fluorescence of individual nuclei was measured (in the FL_2 channel set for log scale) on a FACScan flow cytometry (Becton-Dickinson).

Statistics

Statistical analysis was determined by Student's t-test. Each experiment was performed at least three times, with representative results shown.

RESULTS

DBTC activates the synthesis of genes involved in apoptosis

It has been shown previously that DBTC induced synthesis of RNA (Gennari *et al*., 2000a, see also chapter 3), but the characteristics of the RNA molecules that are produced remained undefined. RNA extracted from freshly isolated rat thymocytes treated with 3 µM DBTC, was reverse transcribed to cDNA and then, using a rat cDNA macro-array kit, hybridized to membranes. Fig. 1 shows the spots visible after exposure of the cells to EtOH

(vehicle control) or to DBTC. As indicated by arrows, in case of organotin-treatment two genes are found to be transcribed, which correspond to *nur77* and glutathione-S transferase (GST).

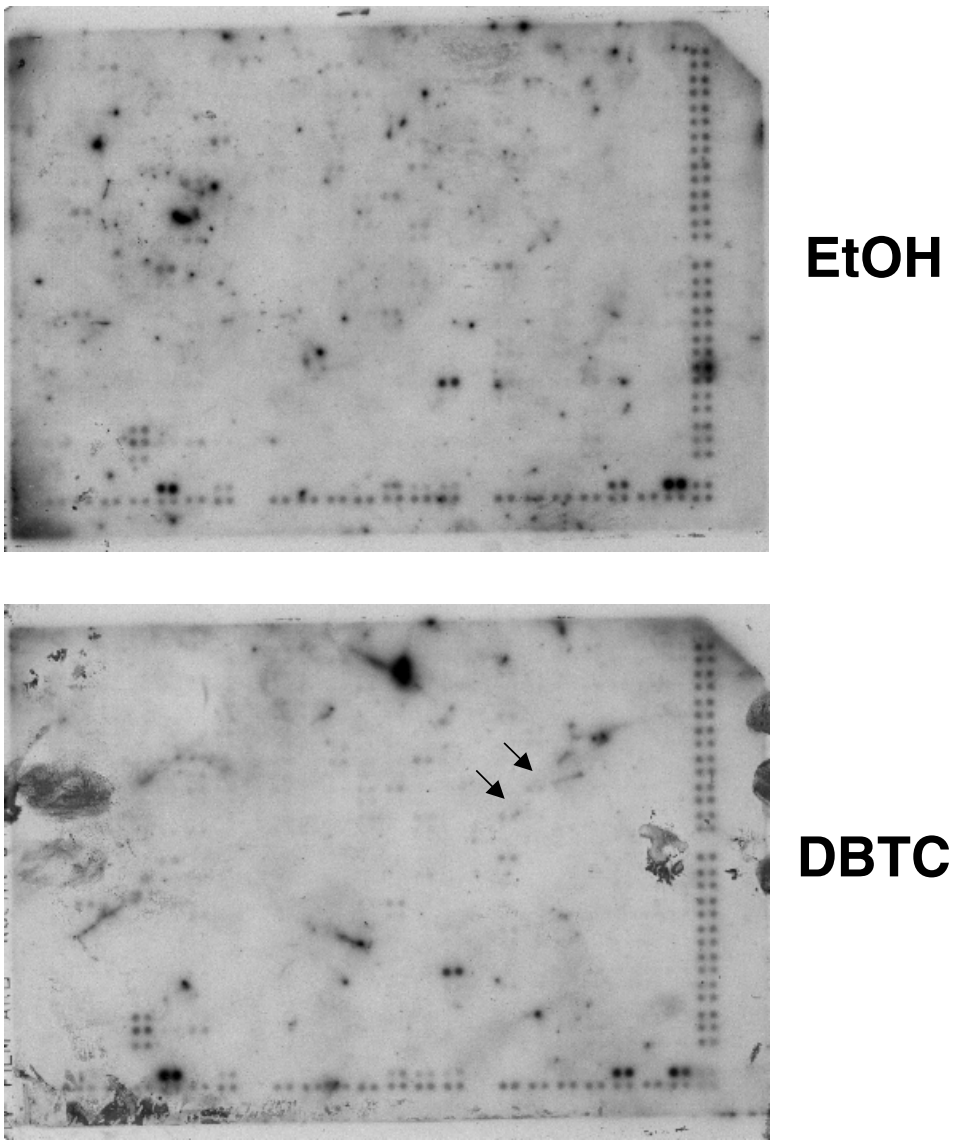

Fig. 1. Atlas rat cDNA expression array membranes. ^{32}P-labeled probes were prepared from 2 µg of total RNA, isolated from rat thymocytes treated for 30 min with EtOH as control (A) or with 3 µM DBTC (B). New genes expression induced by DBTC is indicated with arrows.

To confirm the synthesis of these genes, a RT-PCR has been performed. Clearly, gels in fig. 2 indicate a time-related production of both *nur77* (A) and GST (B) by DBTC.

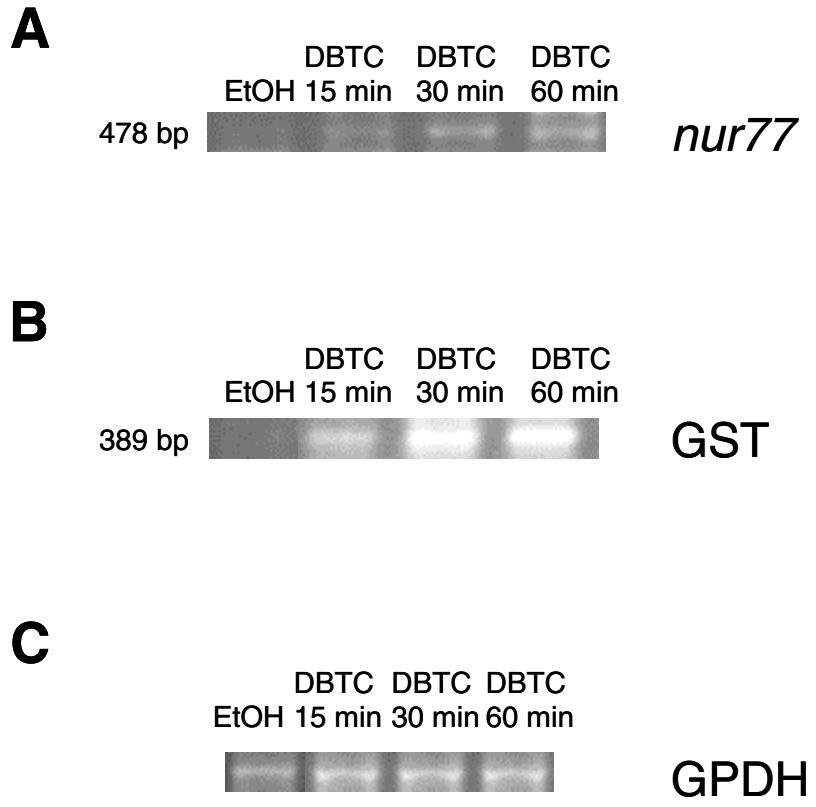

Fig. 2. RT-PCR confirmed the expression of *nur77* and GST. Gels show *nur77* (A), GST (B) and GPDH (C) amplification products from rat thymocytes treated with 3 μM DBTC for 15, 30 or 60 min or with EtOH as control.

Increase of nur77 nuclear proteins by DBTC

A clear increase of the *nur77* nuclear proteins is shown by the EMSA (fig. 3). After 10 min exposure of thymocytes to 3 μM DBTC, followed by 30 min of incubation without compound, a significant activation of *nur77* is present in DBTC-treated cells, compared to controls.

Inhibition of DBTC-induced apoptosis after antisense oligonucleotide treatment

To test whether the presence of *nur77* was linked to organotin-induced DNA-fragmentation, we blocked the transcription of *nur77* with pretreatment of thymocytes with antisense oligonucleotide followed by 10 min exposure to DBTC, and then by overnight incubation in presence of oligonucleotide. Detection of apoptosis by flow cytometry revealed that the percentage of apoptotic nuclei significantly decreased when activation of *nur77* was inhibited (fig. 4), indicating for the first time an important role of *nur77* in DBTC-induced apoptosis.

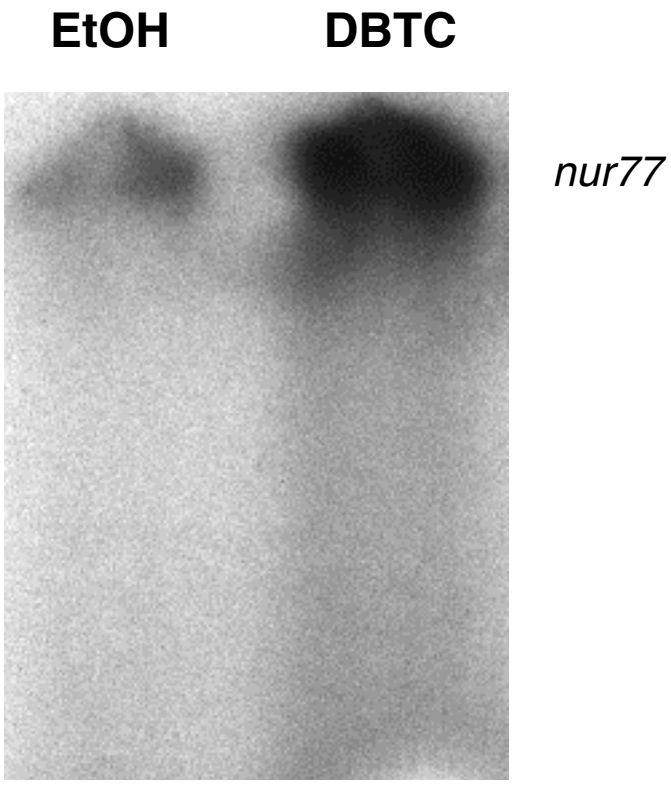

Fig. 3. DBTC induced *nur77* activation. Four µg of nuclear extracts were analysed by electrophoretic mobility shift assay (EMSA), with a ^{32}P-labeled DNA probe detecting the expression of *nur77*.

DISCUSSION

Previously, it has been shown that the organotin compound DBTC stimulates the synthesis of RNA and heat shock protein (HSP) in rat thymocytes. RNA synthesis occurred especially in the small high-density $CD4^+CD8^+$ subset, and this population was found to be also the one vulnerable to induction of apoptosis by DBTC and TBTC (Gennari *et al*.,

2000a, see also chapter 3). Although recent data demonstrated that organotin-induced apoptosis requires *de novo* gene expression (Gennari *et al*., 2000a, see also chapter 3), nothing is known about the molecular pathways that mediate this response.

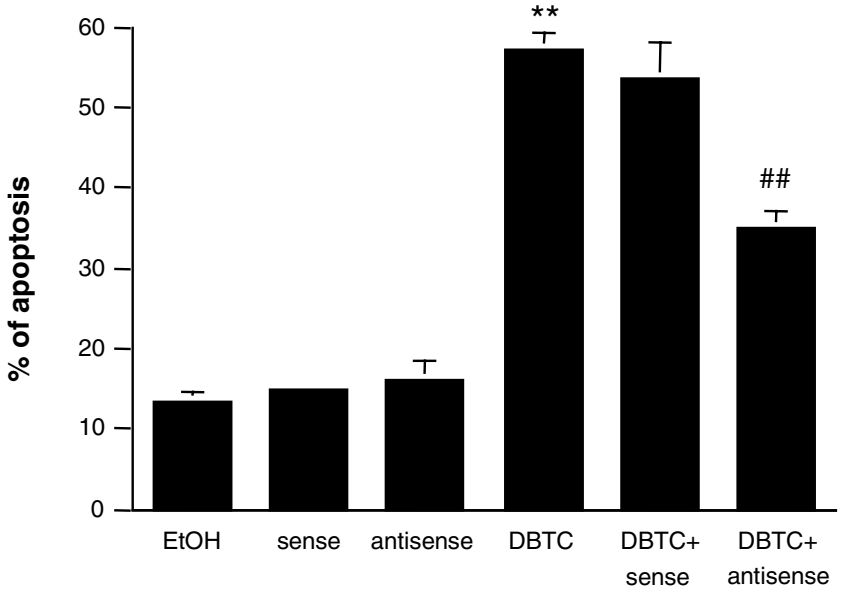

Fig. 4. DBTC-induced apoptosis can be reduced by *nur77* antisense oligonucleotides. Cells were treated as described in Materials and Methods. Values are means ± SD of three separate experiments. ***p* <0.01 vs. control treated cells; ##*p* <0.01 vs. DBTC treated cells.

In order to characterize newly activated genes that are translated after short exposure of thymocytes to DBTC, we first used the macro array cDNA kit, and then different techniques to confirm the obtained data. Two genes, glutathione S-transferase (GST) and *nur77* have been identified, and both of them are known to be involved in the apoptotic process by various stimuli (Flomerfelt *et al*., 1993; Winoto, 1994).

GST is an antioxidant defense enzyme involved in cellular detoxification (Mannervik and Danielson, 1988) and it appeared to be regulated by reactive oxygen species (ROS) activation (Rushmore *et al.,* 1991). During apoptosis, an increase of ROS production induces an increase of GST-levels. Thus, GST activation is a clear sign of cellular

oxidative stress, rather then a necessary step in the apoptotic pathway (Flomerfelt *et al*., 1993).

Nur77 is a member of the steroid/thyroid hormone receptor superfamily, which encodes a number of ligand-dependent transcription factors. It has been shown to be expressed in TCR-mediated DNA fragmentation (Liu *et al*., 1994; Winoto, 1994).

In contrast to GST, *nur77* is considered to be a required transcription factor for the initiation of apoptosis (Liu *et al*., 1994; Winoto, 1994). Indeed, our findings showed that rat thymocytes treated with antisense oligonucleotides, which block the transcription of *nur77,* significantly reduced DBTC-induced apoptotic nuclei compared to cells treated only with the organotin compound. These data clearly indicate that DBTC requires the synthesis of *nur77* to initiate the apoptotic process. Although not much is known about the targets of *nur77*, it is interesting to note that the glucocorticoid receptor (GR) activity antagonizes *nur77*-dependent transcription (Philips *et al.,* 1997). Since it has been found that glucocorticosteroids antagonize organotin-induced apoptosis (Zucker *et al.,* 1994), it can be speculated that this effect is due to the repression of *nur77* by glucocorticosteroids.

Furthermore, previous findings demonstrated that transcription of *nur77* is regulated by calcium signals (Woronicz *et al.,* 1995). This is in accordance with our earlier results (Gennari *et al.,* 2000b, see also chapter 5), showing the increase of intracellular calcium levels as an initial step in organotin-induced apoptosis.

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Chapter five

ORGANOTINS INDUCE APOPTOSIS BY DISTURBANCE OF [Ca^{2+}] AND MITOCHONDRIAL ACTIVITY, CAUSING OXIDATIVE STRESS AND ACTIVATION OF CASPASES IN RAT THYMOCYTES

ALESSANDRA GENNARI[1,2], BARBARA VIVIANI[1], CORRADO LODOVICO GALLI[1], MARINA MARINOVICH[1], RAYMOND PIETERS[2], AND EMANUELA CORSINI[1]

[1]Laboratory of Toxicology, Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

[2]Research Institute of Toxicology, Immunotoxicology Section, Utrecht University, PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

ABSTRACT

Di-*n*-butyltin dichloride (DBTC) and tri-*n*-butyltin chloride (TBTC) cause thymus atrophy in rodents. At low doses, anti-proliferative modes of action have been shown to be involved, whereas at higher doses apoptosis seems to be the mechanism of thymotoxicity by these chemicals. *In vitro,* a similar concentration-dependency has been observed. The purpose of the present research was to investigate the mechanisms underlying DNA fragmentation induced by these organotin compounds in freshly isolated rat thymocytes. As previously shown for TBTC, also DBTC is able to significantly increase intracellular Ca^{2+} level ($[Ca^{2+}]_i$). The rise in $[Ca^{2+}]_i$, already evident 5 min after treatment, was followed by a dose- and time-dependent generation of reactive oxygen species (ROS) at the mitochondrial level. Simultaneously, organotins induced release of cytochrome *c* from the mitochondrial membrane into the cytosol. ROS production and release of cytochrome *c* were reduced by BAPTA, an intracellular Ca^{2+} chelator, or rotenone, an inhibitor of the electron entry from complex I to ubiquinone, indicating the important role of Ca^{2+} and mitochondria during these early intracellular events. Furthermore, we demonstrated that rotenone prevents apoptosis induced by 3 μM DBTC or TBTC, and in addition that both BAPTA and Z-DEVD FMK (mainly a caspase-3 inhibitor) decreased apoptosis by DBTC (already shown for TBTC).

Taken together these data show that the apoptotic pathway followed by organotin compounds starts with an increase of $[Ca^{2+}]_i$, then continues with release of ROS and cytochrome *c* from mitochondria, activation of caspases, and finally results in DNA fragmentation.

INTRODUCTION

Dialkyltin compounds, such as di-*n*-butyltin chloride (DBTC), are industrially applied as heat stabilizers in PVC plastics, and trialkyltin compounds, such as tri-*n*-butyltin chloride (TBTC), are mainly used as biocides in crop protection and antifouling paints for large ships (Fent, 1996).

In rats, organotin compounds reduce cortical thymocyte number and consequently thymus weight (Seinen and Willems, 1976), and after prolonged exposure suppress T-

cell-mediated immune responses (Seinen and Willems, 1976; Seinen *et al.,* 1977). Low doses of both DBTC and TBTC inhibit immature thymocyte proliferation, whereas high doses of TBTC cause a depletion of thymocytes by apoptosis (Raffray and Cohen, 1993).

Previous kinetic studies have indicated that TBTC is dealkylated to DBTC, and that DBTC is the toxicologically active compound (Snoeij *et al.,* 1988).

In vitro, DBTC and TBTC are cytotoxic as well. At low concentrations, they effectively inhibit DNA synthesis (Seinen *et al*., 1979; Snoeij *et al.,* 1986), affect macromolecular synthesis (Snoeij *et al*., 1986; Gennari *et al*., 2000, see also chapter 3), and disrupt mitochondrial energy metabolism (Cain *et al*., 1977; Snoeij *et al*., 1986). At higher concentrations (1-5 μM) these compounds are known to induce apoptosis (Aw *et al.,* 1990; Raffray and Cohen, 1991; Gennari *et al.,* 1997, see also chapter 2).

The mechanistic studies have already linked a TBTC-induced sustained increase in the cytosolic free Ca^{2+} concentration to a subsequent endonuclease activation and DNA fragmentation (McConkey *et al*., 1989; Chow *et al*., 1992). On the contrary, due to the previously unnoticed potential of DBTC to induce apoptosis, no mechanistic information exists about the apoptotic process elicited by this chemical.

Many of the chemical and physical treatments capable of inducing apoptosis are also associated with oxidative stress, suggesting an active role for reactive oxygen species (ROS) in cell death (Buttke and Sandstrom, 1994). An important intracellular source of ROS are mitochondria. Tri-organotin compounds are well known to disturb mitochondrial activity inhibiting ATP synthesis (Snoeij *et al*., 1987; Marinovich *et al*., 1990), and it has been suggested that also DBTC could affect oxidative phosphorylation of mitochondria in a similar way (Cain *et al*., 1977; Penninks *et al*., 1983). Corsini *et al.* (1996; 1998) demonstrated that alterations of Ca^{2+} homeostasis precede TBTC-induced ROS production at the mitochondrial level in murine keratinocytes.

Furthermore, targeting of mitochondria by TBTC has been shown capable of releasing pro-apoptotic factors, such as cytochrome *c*, which is considered a primary event in the induction of DNA fragmentation (Liu *et al*., 1996; Kroemer, 1997). TBTC induced release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol in Jurkat cells and subsequently activated caspases, which cleave target proteins leading to the irreversible damage of the cell (Stridh *et al*., 1998).

The aim of this chapter was to define in thymocytes the apoptotic pathway elicited by organotin compounds. A particular interest has been given to DBTC, as for this metabolite of TBTC information on the process of DNA fragmentation is lacking.

MATERIALS AND METHODS

Chemicals and solutions

Dibutyltin chloride (DBTC) was obtained from Johnson Matthay Alfa Products (Karlsruhe, Germany), tributyltin chloride (TBTC) from Aldrich (Steinhein, Germany), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di(acetomethyl ester) (DCFH) and 1,1-bis(2-aminophenoxy)ethane-*N,N,N,N'*-tetraacetic acid (BAPTA) from Molecular Probes (Eugene, OR). Fura-2/AM, rotenone, ruthenium red (RR) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). Ribonuclease A (RNase, type I-A, Sigma) was dissolved first in Tris buffer (10 mg/ml) and boiled for 15 min and then diluted in RNase buffer (0.1% sodium citrate, 0.1% Triton X-100) to obtain a final concentration of 0.5 mg/ml. Hank's balanced salt solution (HBSS) was obtained from Sigma and RPMI 1640 (Sigma) was supplemented with 5% fetal calf serum (Sigma), penicillin (100 U/ml), and streptomycin (100 µg/ml). Krebs-Henseleit buffer contained 20 mM HEPES, 5.5 mM glucose, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM KCl, 119 mM NaCl, pH 7.4. Z-DEVD-FMK (mainly a caspase 3 inhibitor) was purchased from Calbiochem (La Jolla, CA, USA) and dissolved in DMSO to obtain the final concentration of 20 µM.

Animals

Four–five weeks old female Wistar rats (Charles River, Calco, Italy) were used for the experiments. Animals were housed three to four per cage over woodchip bedding, and allowed food and water *ad libitum*. Rats were killed with CO_2 and thymi were isolated.

Cell suspensions and incubation

Cell suspensions from thymi were prepared as described by Gennari *et al*. (1997, see also chapter 2). Thymocytes were incubated with different concentrations of DBTC or TBTC dissolved in ethanol absolute (EtOH), or with EtOH as vehicle control (the final EtOH concentration was 0.1%, which is ineffective by itself).

For measurement of intracellular Ca^{2+} and oxidative activity (see below) thymocytes were incubated with different concentrations of DBTC or TBTC (0-5 μM). To calculate the release of cytochrome *c* cells were treated with 3 μM of organotin compounds for 5 min.

To detect apoptosis thymocytes were treated with 3 μM of organotins for 10 min, then washed and incubated overnight at 37°C without compounds.

When necessary cells were pre-treated 30 min with BAPTA (10 μM, in DMSO), 30 min with RR (5 μM, in H_2O), 1 h with rotenone (20 μM, in DMSO) or 30 min with Z-DEVD-FMK (20 μM, in DMSO) before incubation with organotins.

Determination of Ca^{2+} level

Isolated rat thymocytes ($2x10^7$ cells/ml) were loaded with 4 μM Fura-2/AM in buffer A (Krebs-Henseleit buffer supplemented with 2% BSA) for 30 min at room temperature. Cells were then washed and resuspended in buffer A without Fura-2/AM for another 15 min, to allow complete hydrolysis of Fura-2/AM. Just before use, thymocytes ($3x10^6$ cells) were washed with buffer B (Krebs-Henseleit buffer supplemented with 0.2% BSA) and resuspended in the same buffer in a quartz cuvette, where organotins were added. The Fura-2/AM ratio signal was measured in a Perkin Elmer LS 50 B double-wavelength fluorometer and calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkievicz *et al.* (1985).

Oxidative activity

Thymocytes ($5x10^6$ cells/ml) were washed once with HBSS containing 2% BSA and loaded with 10 μM DCFH (stained) or DMSO (unstained) for 1 h at 37°C. Then cells were washed once in HBSS and oxidative activity was assessed as follows. Ten million cells in quartz cuvettes containing 2 ml HBSS were treated with different concentrations of DBTC, TBTC or EtOH as control. ROS production was measured as DCFH oxidation by the intensity of the emission of 525 nm fluorescence excited by 503 nm (Perkin Elmer LS 50B), at time 0, 5, 10, 15 and 30 min following treatment in both stained and unstained cells. Results are expressed as the change in fluorescence (in arbitrary units, AU) calculated as

$$AU = [I_{stained} - I_{unstained}]\ t_x - [I_{stained} - I_{unstained}]\ t_0,$$

where I represents the intensity of fluorescence.

When necessary, cells were treated during loading period with BAPTA, RR or rotenone.

Cytochrome c release

After treatment with organotins, and eventually with BAPTA or rotenone, thymocytes ($5x10^6$ cells/ml) were washed once in 5 ml ice-cold phosphate buffered saline (PBS). Cells were then centrifuged and the pellet was resuspended in 200 µl of ice-cold buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol [DTT], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), supplemented with protease inhibitors (1:50 protease inhibitor cocktail, Sigma). After being kept on ice for 15 min, cells were lysed by passing 15 times through a G22 needle. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatants were further centrifuged at $4x10^4$ g for 30 min at 4°C in a table top ultracentrifuge.

Protein concentration was calculated using the Bio-Rad Protein Assay with albumin as the standard.

Western blotting

Protein extracts (20 µg) were loaded onto a 18% SDS-polyacrylamide gel and electrophoresed at 120V, then transferred to PVDF membranes (NEN, Boston, MA) at 250 mA for 1 h. Membranes were blocked in 5% non-fat dried milk and then incubated with a rabbit anti-cytochrome *c* polyclonal IgG antibody (1:1000 diluted in 5% non-fat dried milk, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with an anti-rabbit IgG alkaline phosphate (AP)-conjugate antibody (1:50000 diluted in 5% non-fat dried milk, Sigma) for 1 h at room temperature, and visualized by CDP-star (NEN).

The Western Blot image was acquired with a Nikon CCD video camera module (Nikon, Melville, NY). The optical density of the bands was calculated, and peak area of a given band was analyzed by means of the Image 1.61 program for digital image processing (Wayne Rasband, Research Service Branch, NIH, MD).

Apoptosis detection

Detection of apoptosis by flow cytometry with PI as fluorescent indicator was done essentially as described by Nicoletti *et al*. (1991). Briefly, after incubation with the test compounds, $5x10^6$ cells/ml were centrifuged and resuspended in 1 ml PBS. Of this suspension 200 µl was incubated for 30 min with 0.5 ml RNase (0.5 mg/ml) at room temperature. Then, 0.5 ml PI was added (5 µg/ml, in PBS) and the fluorescence of individual nuclei was measured (in the FL_2 channel set for log scale) on a FACScan flow cytometry (Becton-Dickinson, Italy).

Statistics

Statistical analysis was determined by Student's t test or Dunnet's multiple comparison test, after analysis of variance. Each experiment was performed at least three times, with representative results shown.

RESULTS

DBTC increases the level of Ca^{2+}

It has been previously shown (McConkey *et al.,* 1989; Chow *et al.,* 1992) that TBTC is able to increase cytosolic free Ca^{2+} concentration, but not much is known about the influence of DBTC on Ca^{2+} level. Freshly isolated rat thymocytes loaded with Fura-2/AM, were incubated with increasing concentrations (0-5 µM) of DBTC, and then analyzed for cytoplasmic Ca^{2+} concentration. As shown in fig. 1, after addition of DBTC was detectable an immediate rise in $[Ca^{2+}]_i$, that reached a plateau level in 5 min for all the concentrations tested. The plateau levels were plotted in the graphic, and clearly showed a dose-dependent intracellular Ca^{2+} increase which was statistically significant from the concentration of 3 µM.

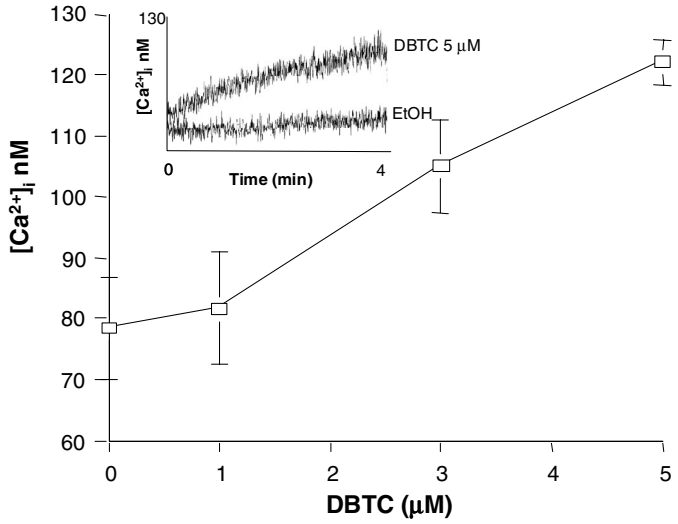

Fig. 1. DBTC induces a dose-dependent increase in $[Ca^{2+}]_i$. Cells were loaded with fura-2/AM, then incubated with increasing concentrations (0-5 μM) of DBTC and after 5 min $[Ca^{2+}]_i$ was measured as described in Materials and Methods. Each value represents mean ± SD of three experiments. Statistical analysis was determined by Student's t test.

Organotin compounds induce reactive oxygen species production

Because alterations in Ca^{2+} homeostasis may induce oxidative stress, we tested whether organotin compounds may affect reactive oxygen species (ROS) generation. As shown in fig. 2A, DBTC was able to induce ROS production in rat thymocytes in a time- and dose-dependent manner. A significant increase of ROS was observed starting from 5 min of treatment. Also TBTC induced a time- and dose-dependent production of ROS (fig. 2B), and it was at least three times more potent then DBTC. Indeed significant increase in ROS was observed from 3 μM DBTC, whereas TBTC induced already a significant ROS production at 1 μM.

We then hypothesized that organotin-induced increase in $[Ca^{2+}]_i$ preceded ROS generation at the mitochondrial level. To assess the role of Ca^{2+} and mitochondria in organotin-induced ROS release, cells were pre-treated with non-cytotoxic concentrations of the intracellular Ca^{2+} chelator BAPTA, or with the mitochondrial electron transport

chain-inhibitor rotenone. Both treatments significantly reduced DBTC- and TBTC-induced production of ROS. At the concentration tested, BAPTA and rotenone reduced respectively of 41% and 28% DBTC-induced ROS (fig. 3A), and of 68% and 87% TBTC-induced ROS production (fig. 3B).

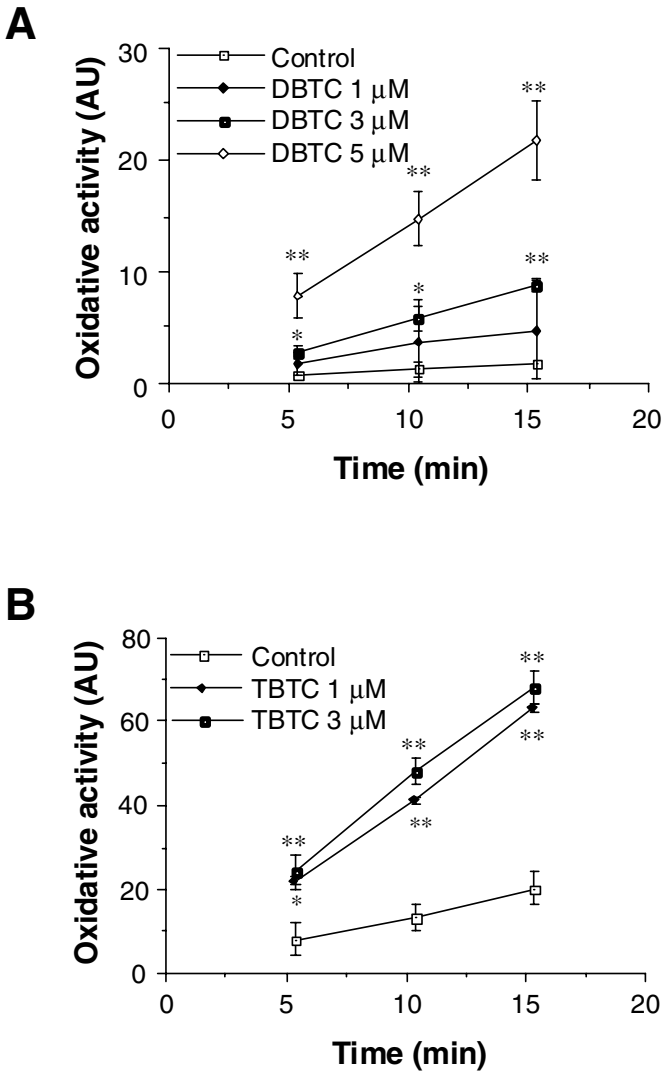

Fig. 2. DBTC (A) and TBTC (B) induce cellular oxidative activity. ROS generation was measured by DCFH oxidation, as indicated in Materials and Methods. Graphics represent the mean ± SD of three experiments. Statistical analysis was determined by Dunnett's t test with $*p < 0.05$ and $**p < 0.01$ vs. EtOH treated cells.

To further confirm the link between $[Ca^{2+}]_i$ and generation of ROS at mitochondria level, thymocytes were pre-treated with ruthenium red (RR), an inhibitor of mitochondrial Ca^{2+} channels. Also in this case, the combination of RR with organotin compounds significantly decreased the release of ROS of 50% for DBTC and 65% for TBTC (table 1), confirming mitochondria as the primary source of organotin-induced ROS production.

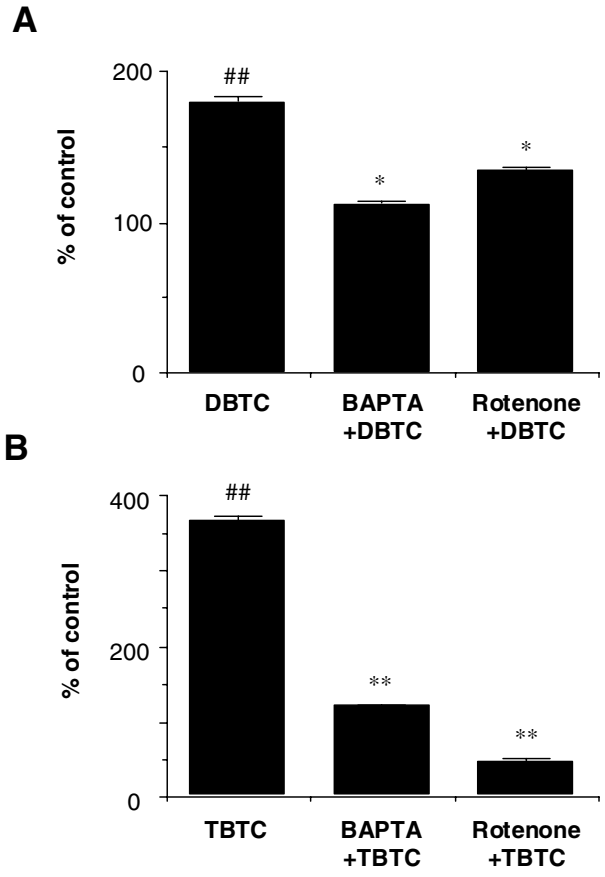

Fig. 3. BAPTA and rotenone prevent ROS production in thymocytes treated with organotin compounds. Cells were first treated for 30 min with 10 μM BAPTA, or for 1 h with 20 μM rotenone or with DMSO as vehicle control, and then 3 μM DBTC (A) or 3 μM TBTC (B) were added. Oxidative activity was measured 15 min after organotin treatment. The arbitrary units (AU) of fluorescence in the control group was 7±1. Each value represents mean ± SD of three experiments. Statistical analysis was determined by Dunnett's t test with $*p < 0.05$, $**p < 0.01$ vs. cells treated with DBTC/TBTC and $\#\# p < 0.01$ vs. control treated cells.

Table 1- Ruthenium red (RR) prevents organotin-induced cellular oxidative activity.

Treatment	Oxidative activity (% of relative control)
DBTC	212 ± 3##
DBTC + RR	110 ± 2*
TBTC	312 ± 9##
TBTC + RR	175 ± 11#,*

Cells were pre-treated for 30 min with 5 µM RR and then for 15 min with 3 µM DBTC or 3 µM TBTC. The arbitrary units (AU) of fluorescence in the control group was 13±1. Each value represents mean ± SD of three experiments. Statistical analysis was determined by Dunnett's t test with $*p < 0.05$ vs. cells treated with DBTC/TBTC and $\#p < 0.05$, $\#\#p < 0.01$ vs. control treated cells.

DBTC and TBTC induce cytochrome c release

It has been previously shown in Jurkat cells that TBTC induced a rapid loss of mitochondrial membrane potential, which is associated with the release of cytochrome *c*, important for subsequent apoptosis (Stridh *et al*., 1998). Here, we tested whether also DBTC, as TBTC, induced the release of cytochrome *c* from the mitochondrial membrane into the cytosol of rat thymocytes. Cells were treated with 3 µM DBTC or TBTC for 5 min. As shown by Western blot analysis of cytosolic extracts (fig. 4), both compounds induced the release of cytochrome *c* (12 kDa). Interestingly, the release of cytochrome *c* induced by organotins could be modulated by pre-treating thymocytes with BAPTA or rotenone (fig. 4), indicating a role for Ca^{2+} and ROS in organotin-induced cytochrome *c* release. By densitometric analysis, the decrease in DBTC-induced cytochrome *c* release by BAPTA or rotenone was of 32% and 27% respectively, while in case of TBTC was of 52% and 55% respectively.

Reduction of apoptosis induced by DBTC and TBTC in presence of inhibitors

To link the early intracellular events induced by organotin compounds with later induction of apoptosis, thymocytes were pre-treated with BAPTA or rotenone and DNA

fragmentation was assessed by flow cytometry. Relatively to DBTC, the addition of BAPTA or rotenone inhibited DBTC-induced apoptosis (fig. 5), indicating the importance of $[Ca^{2+}]_i$ and mitochondria as events leading to cell death. For TBTC the protective role of BAPTA has been already shown by others (Aw *et al*., 1990), but we found that TBTC-induced apoptosis could also be prevented by rotenone (fig. 5), demonstrating the key role of mitochondria in the apoptotic process.

We then used Z-DEVD FMK to inhibit caspase 3 activation, in order to characterize the apoptotic pathway activated by DBTC. As shown in table 2, apoptosis induced by 3 µM DBTC was down-regulated by 30 min pre-treatment of the cells with Z-DEVD FMK (already shown for TBTC, Stridh *et al.*, 1998).

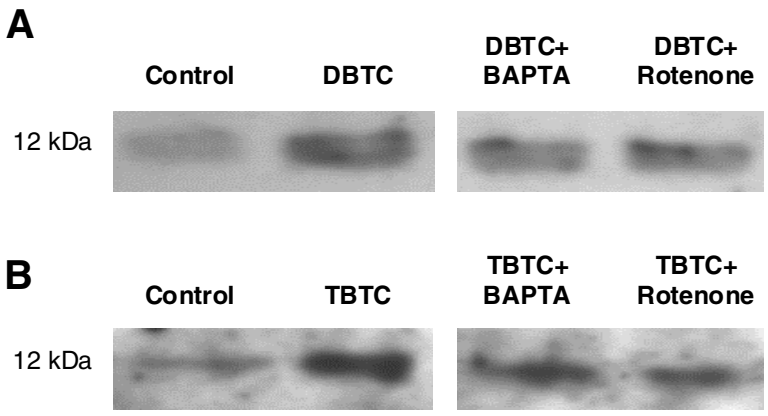

Fig. 4. DBTC and TBTC induce a rapid cytochrome *c* release. The presence of cytochrome *c* (12 kDa) in cytoplasmic extracts was measured by Western blot with an anti-cytochrome *c* antibody, as described in Materials and Methods. Sample extracts were prepared from thymocytes treated with EtOH (control), 3 µM DBTC (A) or 3 µM TBTC (B) for 5 min. BAPTA (10 µM, for 30 min) or rotenone (20 µM, for 1 h) pre-treatment, followed by addition of 3 µM DBTC or TBTC for 5 min, was used to demonstrate the role of Ca^{2+} and ROS in organotin-induced cytochrome *c* release.

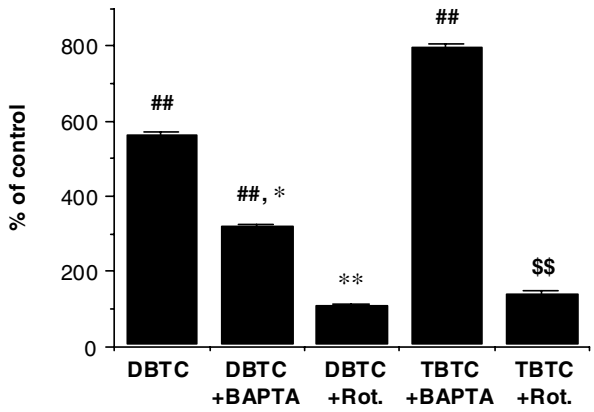

Fig. 5. Organotin-induced apoptosis could be modulated by BAPTA and rotenone treatment. Flow cytometry (PI staining) was used to quantify apoptosis induced by 3 μM DBTC or 3 μM TBTC, as described in Materials and Methods. The amount of apoptotic nuclei is indicated as percentage of relative control. The percentage of apoptotic nuclei in the control group was 10±1%. Values are means ± SD of three separate experiments. Statistical analysis was determined by Dunnett's t test with **p* <0.05 vs. cells treated with DBTC; ***p* <0.01 vs. cells treated with DBTC; $$*p* <0.01 vs. cells treated with TBTC; ##*p* <0.01 vs. control treated cells.

Table 2- Z-DEVD FMK reduces apoptosis induced by 3 μM DBTC.

Treatment	**Apoptosis (% of relative control)**
DBTC	560 ± 5##
DBTC + Z-DEVD FMK	380 ± 3##,*

Flow cytometry was used to quantitatively measure DNA fragmentation, as described in Materials and Methods. The percentage of apoptotic nuclei in the control group was 10±1%. Values are means ± SD of three separate experiments. Statistical analysis was determined by Student's t test with **p* < 0.05 vs. cells treated with DBTC and ##*p* < 0.01 vs. control treated cells.

DISCUSSION

Here, we demonstrate the important role of $[Ca^{2+}]_i$ and mitochondria during apoptosis induced by DBTC and TBTC in rat thymocytes. Previously, it has been shown that one of the early events in the apoptotic cell death induced by TBTC is the rise in intracellular Ca^{2+} concentration (Aw *et al*., 1990). We have found that also DBTC is able to increase the $[Ca^{2+}]_i$, at concentrations greater than 1 μM.

Based on previous results (Corsini *et al.*, 1996; 1998) obtained in murine keratinocytes, we hypothesized that a disturbance of the Ca^{2+} homeostasis may initiate organotin-induced oxidative stress in thymocytes as well. High cytoplasmic Ca^{2+} levels can cause an increased mitochondrial Ca^{2+} uptake and disruption of mitochondrial Ca^{2+} equilibrium, which results in ROS formation (Chacon and Acosta, 1991) due to stimulation of electron flux along the electron transport chain. Indeed both DBTC and TBTC were able to induce generation of ROS in a time- and dose-dependent manner. Important to note is that TBTC was more potent than DBTC in increasing oxidative activity.

To correlate Ca^{2+} and mitochondria in ROS release, rat thymocytes were pre-treated with the Ca^{2+} chelator BAPTA, the electron transport chain-inhibitor rotenone as well as with RR, an inhibitor of mitochondrial Ca^{2+} channels. The presence of these inhibitors resulted in a significant reduction of organotin-induced oxidative activity, indicating that the uptake of Ca^{2+} at the mitochondrial level is necessary for the generation of reactive oxygen species induced by organotins.

It is known that mitochondrial cytochrome *c* release from the inner membrane into the cytosol is an early common event in the induction of apoptosis by multiple agents, and that cytochrome *c* release is linked to caspase activation and subsequent DNA fragmentation (Stridh *et al*., 1998). Like previously shown for TBTC (Stridh *et al*., 1998), we demonstrate that also DBTC is able to induce the release of cytochrome *c*. The addition of BAPTA or rotenone to the cells before the organotin compounds modulated cytochrome *c* release, suggesting that the increase of intracellular Ca^{2+}, ROS release and transport of cytochrome *c* into the cytosol are early and functionally correlated events in the pathway leading to DNA fragmentation induced by organotins. It is still difficult to temporally separate the effect of organotin compounds on mitochondria and the release of

cytochrome *c* (both events occurring within few minutes). Our results, based on the modulatory effect of rotenone, led us to the proposal that ROS production precedes cytochrome *c* release.

The subsequent step has been the evaluation of the possible involvement of caspases during DNA fragmentation, knowing that a link between release of cytochrome *c* by TBTC and activation of caspase 3 has been shown by Stridh *et al.* (1998) on Jurkat cells. Present data indicate that inhibition of caspase 3 reduced the extent of DBTC-induced apoptosis, confirming also for this compound the crucial role of the caspase family in the activation of apoptotic cell death.

Thus, we conclude that both organotin compounds DBTC and TBTC initiate an increase of $[Ca^{2+}]_i$, causing the generation of ROS and release of cytochrome *c* by mitochondria. As a result caspases are activated, cleaving defined target proteins leading to an irreversible apoptotic damage of the cell. The influx of Ca^{2+} may be caused by disruption of membrane or cytoskeletal functioning. This has already been demonstrated for TBTC (Chow and Orrenius, 1994) whereas DBTC has been found capable of cytoskeleton-mediated capping of T cell receptor and CD8 molecules on thymocytes (Pieters *et al*., 1995).

Previously, it has been shown (Gennari *et al.,* 2000, see also chapter 3) that both DBTC and TBTC increased RNA synthesis in small high-density $CD4^+CD8^+$ thymocytes at similar concentrations as used in the present study. Whether the early mitochondrial effects and increased RNA synthesis are functionally linked, and whether the synthesized RNA molecules play a role in the fragmentation of DNA, is subject of research.

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Chapter six

THYMUS-INDEPENDENT INHIBITION OF HAPTEN-INDUCED IMMUNOSTIMULATION BY THE ORGANOTIN COMPOUND DBTC

ALESSANDRA GENNARI, PAUL KOENEN, AND RAYMOND PIETERS

Research Institute of Toxicology, Immunotoxicology Section, Utrecht University,
PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

ABSTRACT

Previous studies have shown that thymus atrophy caused by the organotin compound di-*n*-butyltin dichloride (DBTC) is the consequence of inhibition of immature thymocyte proliferation. After prolonged exposure, the number of peripheral T cells as well as T cell-mediated immune functions was found to be diminished, and this suppression of peripheral immune response was hypothesized to be the result of reduced seeding of mature thymocytes from the thymus to the peripheral immune organs. The apparent thymus selectivity of organotin compounds, however, contradicted *in vitro* data, showing that DBTC inhibited proliferation and induced apoptosis in both thymocytes and splenocytes. Thus, we argued that DBTC may affect both thymocytes and T cells but their vulnerability depends on the state of activation. To examine this, the effect of a single oral dose of DBTC on a hapten (DNCB)-induced immunostimulation was assessed. Rats were sensitized by painting 1% (w/v) DNCB on the ears, and the number of DNP-specific IgG forming cells in the auricular lymph nodes were determined by ELISPOT-assay. Results demonstrated that DBTC not only reduced the number of thymocytes, but also the number of antibody forming cells. The fact that IgG production is T cell-dependent, combined with the fast kinetics of reduction (3 days after DBTC dosing), indicates that DBTC has a direct effect on peripheral T cell activation.

This study shows for the first time that organotin compounds are not thymus-selective, but rather affect activation of T cells in general.

INTRODUCTION

The organotin compounds that have been most extensively studied are dialkyltin compounds, such as di-*n*-butyltin dichloride (DBTC), used primarily as stabilizers for polyvinyl chloride plastics, and trialkyltin compounds, such as tri-*n*-butyltin chloride (TBTC), which are widely used as biocides (Fent, 1996).

In vivo, both of these organotins are known to induce thymus atrophy initiated by a rapid reduction of the proliferation of thymocytes in rats (Penninks and Seinen, 1987; Vos and Penninks, 1987; Snoeij *et al.*, 1988). A single oral dose of DBTC induced a marked decrease of the number of proliferating, immature $CD4^{-}CD8^{+}TcR\alpha\beta^{-/low}$ $CD4^{+}CD8^{+}TcR\alpha\beta^{-/low}$ thymoblasts within 24 to 48 h. The large population of small, non

dividing $CD4^+CD8^+$ thymocytes gradually decreased and was maximally reduced at day 4 to 5 after dosing, when the number of thymoblasts had returned to control level again (Snoeij *et al.,* 1988; Pieters *et al.,* 1992). Within 7-9 days thymus weight and total thymocyte count were back at the control level. No affection in the spleen weight was observed (Snoeij *et al.,* 1988).

From these data it was concluded that organotin compounds selectively inhibit immature thymocyte proliferation. The selective effect on thymoblasts would generate fewer small cortical thymocytes, a marked depletion of cortical thymocytes and profound reduction in thymus weight (Penninks *et al*., 1985; Vos and Penninks, 1987; Snoeij *et al*., 1988; Pieters *et al.,* 1992). The decrease in the number of peripheral T cells, and the suppression of T cell dependent immune functions after prolonged exposure (longer than 2 weeks) to organotin compounds, were considered to be the result of this primary effect on the thymus.

Although *in vivo* findings appeared to reveal a selective effect of DBTC on immature thymocyte proliferation, parallel *in vitro* studies demonstrated a general anti-proliferative effect of the organotin compound. Notably, DBTC was found to inhibit the mitogen-induced proliferation of splenic T and B lymphocytes as well as the spontaneous and mitogen-induced proliferation of thymocytes (Seinen *et al*., 1979). Moreover, thymocytes and peripheral spleen cells were equally sensitive to apoptosis-inducing concentrations of DBTC (1-5 μM) (Gennari *et al*., 1997, see also chapter 2). This discrepancy between *in vivo* findings, indicative of a thymus-selective effect, and the *in vitro* data, showing that DBTC has a general anti-proliferative and apoptotic activity, has complicated the search for the mechanisms involved in organotin-induced thymus-atrophy.

Here, we aimed to investigate whether organotin compounds have a direct effect on peripheral lymphocyte activation. Therefore, the influence of a single oral dose of DBTC on a epidermally elicited, hapten (2,4-dinitro-1-chlorobenzene, DNCB)-specific induced immunostimulation was assessed.

MATERIALS AND METHODS

Chemicals

DBTC, obtained from Johnson Matthay Alfa Products (Karlsruhe, Germany), was dissolved in ethanol absolute (EtOH) (Merck, Darmstadt, Germany) and further diluted in corn oil (250 ml per rat) to a final EtOH concentration of 5%. 2,4-dinitro-1-chloro-benzene (DNCB) was obtained from Sigma (St. Louis, MO, USA).

For ELISPOT assays, Immobilon-P membranes (Immobilon PVDF Transfer, Millipore, Etten-Leur, The Netherlands), dinitrophenyl human serum albumin (DNP-HSA) (Sigma), bovine serum albumin (BSA, fraction V), and alkaline phosphatase-conjugated goat-anti-rat IgG antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) were used.

Animals and treatment

Male Wistar-derived rats (U:Wu) were bred under specific pathogen-free conditions and housed under standard conditions (room temperature ±20°C, relative humidity ±55%). Rats were 4-5 weeks of age at the start of the experiments.

Fifty µl of a solution of DNCB (1% w/v in a 4:1 mixture of acetone and olive oil) was applied on the dorsum of the ears on 3 consecutive days. Twenty four hours after the last application, animals received a single oral dose of 0 or 20 mg DBTC/kg body weight in corn oil. On day 7, rats were killed by decapitation and thymi, spleens and auricular lymph nodes (ALN) were isolated. Single cell suspensions were then prepared from thymi and ALN and used for further analyses.

Cell suspensions

ALN and thymi were minced to prepare single-cell suspensions and cells were collected in ice-cold PBS (supplemented with 1% BSA in case of lymph nodes). After a washing step, cells were counted by a Coulter counter ZM (Coulter Electronics, Luton, UK). In case of thymocytes, numbers of small cells (diameter between 4.3 µM and 6.8 µM) and large cells (diameter over 8.6 µM) were determined as described before (Snoeij *et al.*, 1988) by varying the threshold values on the Coulter counter.

ELISPOT assay

The assay was performed as previously described (Schielen *et al.,* 1995). Briefly, $5x10^5$ cells/ml in PBS/1% BSA were incubated (37°C, 4 h) in wells containing DNP-HSA-coated (overnight, 4°C) immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of alkaline phosphatase-conjugated goat-anti-rat IgG antibody at 4°C, overnight while shaking. After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium as substrate. Specific antibody forming cells (AFC) per 10^6 cells were then calculated from spot numbers counted with the aid of a stereomicroscope.

Statistics

Preceding statistical analysis, the number of AFC/10^6 cells was transformed to log10 values to homogenize variance. Statistical analysis was determined by Student's t test.

RESULTS AND DISCUSSION

In the present study, we tested whether the anti-proliferative effect of the organotin compound DBTC is solely thymus-specific or whether DBTC is capable of directly inhibiting proliferation of peripheral lymphocytes as well. For this purpose, rats were immunosensitized by painting the allergen DNCB on the ears on 3 consecutive days (Kimber *et al*., 1989). One day after the last application of DNCB, when proliferation in the draining lymph node is known to be high and restricted to the T cell harbouring paracortical region of the lymph node (Kimber *et al*., 1989), an anti-proliferative dose of DBTC (20 mg/kg body weight) was given by gavage. This dose caused a significant decrease of the relative weight of the thymus on day 7 (Fig. 1A). The number of both small and large thymocytes (Fig. 2) was reduced as well, to around 50% and 45% respectively. No loss in body weight gain (not shown) or changes in spleen weight (Fig. 1B) were observed. These findings are in agreement with previous data (Snoeij *et al*., 1988; Pieters *et al.,* 1992) and suggestive of a thymus selective effect of DBTC.

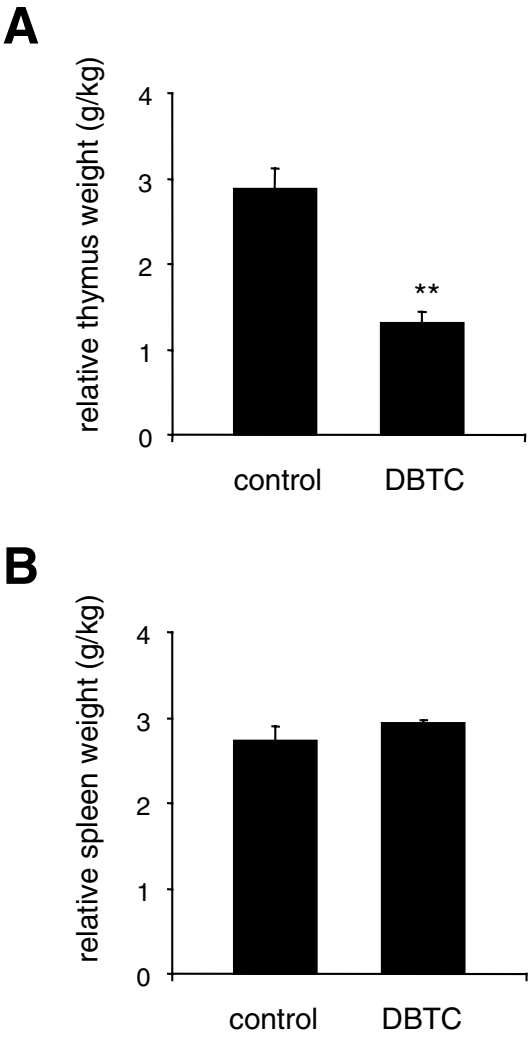

Fig. 1. Relative thymus (A) and spleen (B) weight isolated from rats first sensitized with DNCB (1%w/v) and then orally treated with 20 mg DBTC/kg body weight. The average ± SD of four animals per treatment is shown. $^{**}p < 0.01$.

But, most interestingly, assessment of the hapten-induced immunostimulation showed that the total number of ALN-cells, as well as the number of DNP-HSA-specific IgG forming cells per whole lymph node, were also significantly ($p<0.03$ and $p<0.05$ respectively) decreased in DBTC-treated rats (Table 1).

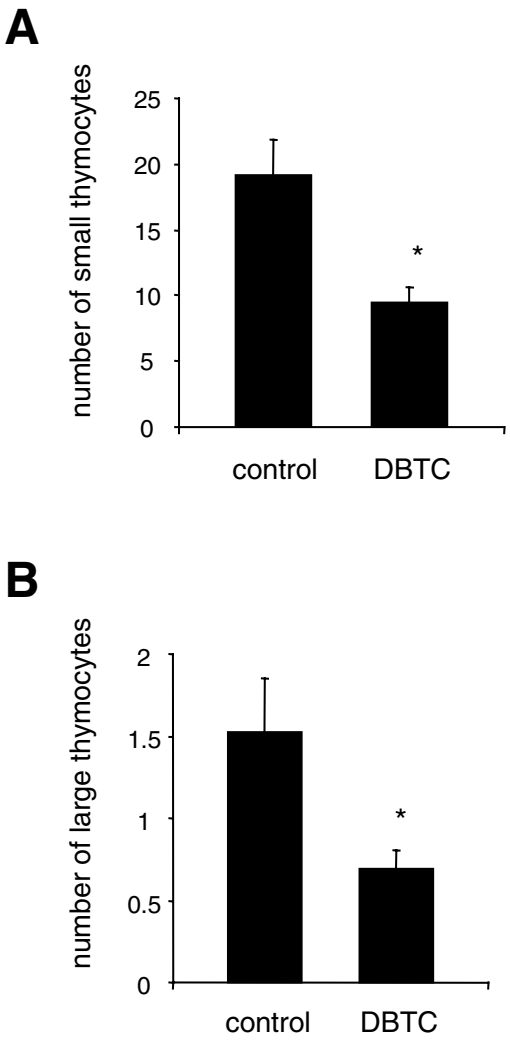

Fig. 2. Number of small (A) and large (B) thymocytes after treatment with 20 mg DBTC/kg body weight. The average ± SD of four animals per treatment is shown. *$p < 0.05$.

After the time period between dosing with DBTC and reading out the response to DNCB (3 days) thymus atrophy is not yet maximal. This period is also too short to explain a diminishment of peripheral T cells from a result of decreased seeding of recent thymic emigrants into the periphery. Thus, it can be concluded that DBTC directly affects the proliferation of lymphocytes in peripheral lymphoid organs.

The number of antibody forming cells per 10^6 was also reduced but not significant (p<0.06), indicating that the relative number of B cells was less affected than the total

number of lymph node cells and the absolute number of B cells. It is tempting to suggest, from these data, that DBTC primarily affects proliferation of T cells, and that the reduction of antibody formation is secondary to an inhibitory effect on T cells. In fact in the presently used method, proliferation on the day after the last application of DNCB has been shown to be restricted to T cells. However, also proliferation of total spleen cells induced by LPS (Seinen *et al.,* 1979) and of tonsilar B cells with Staphylococcus aureus (De Santiago *et al*., 1999), has been shown to be inhibited by DBTC *in vitro*. This suggests that even B cells, providing that they are activated, may be sensitive to DBTC.

Table 1. Number of draining auricular lymph nodes and AFC-IgG after DBTC (20 mg/kg body wt.) treatment.

Treatment	**Cells count ($1x10^6$)**	**AFC-IgG/ lymph node**	**AFC-IgG ($1x10^6$)**
Control	29.4 ± 4.3	3.3 ± 0.4	1.8 ± 0.3
DBTC	15.8 ± 2.0*	2.6 ± 0.2*	1.4 ± 0.2

*$p < 0.05$, compared to controls.

The reason why until now the general anti-proliferative capacities of DBTC have been unnoticed, is due to the fact that the compound has never been tested directly on an activated immune system. All indications of an immunosuppressive state induced by thymotoxic organotins that were previously noticed, were obtained in rats dosed for 2 weeks or longer (Seinen *et al.,* 1977; Vos *et al*., 1984). The need for activation also applies to thymocytes, as it has been shown that the inhibitory effect of DBTC was dependent on the proliferation rate of thymocytes, and that PHA-stimulated thymocyte subsets are more sensitive to the anti-proliferative effect of DBTC (Pieters *et al.,* 1994).

The present findings that DBTC, and possibly also other thymotoxic organotin compounds, have a direct thymus-independent effect on T cell-dependent immune

functions, may have important implications for studying the mechanisms of organotin-induced immunosuppression.

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Chapter seven

GENERAL DISCUSSION

ALESSANDRA GENNARI

Research Institute of Toxicology, Immunotoxicology Section, Utrecht University,
PO Box 80.176, NL-3508 TD, Utrecht, The Netherlands

The aim of this thesis was to investigate whether apoptosis is responsible for the thymus atrophy induced by organotin compounds at anti-proliferative doses, whether DBTC is able to induce apoptosis in vitro, and to define the mechanisms involved in DBTC- and TBTC-DNA fragmentation.

Moreover, it was tested whether organotin compounds have a selective effect on the thymus or rather have a general anti-proliferative effect.

The organotin compounds di-*n*-butyltin dichloride (DBTC) and tri-*n*-butyltin chloride (TBTC) are known to cause thymus atrophy in rats (Seinen and Willems, 1976; Seinen *et al.*, 1977; Vos *et al*., 1984). Initially, these chemicals inhibit the proliferation of large immature thymoblasts, leading to a reduction in the population of $CD4^+CD8^+$ thymocytes, which are the descendants of the initially affected thymoblasts (Snoeij *et al.,* 1988; Pieters *et al.,* 1992).

The aim of the second chapter of this thesis was to examine whether apoptotic cell death is involved in this thymus atrophy. Results showed that neither of the tested DBTC or TBTC induced apoptosis in thymi of animals exposed to a single oral dose (15 mg/kg), which is known to inhibit thymocyte proliferation. In contrast, parallel *in vitro* studies revealed that a short exposure to relatively high concentrations (3-5 μM) of organotins induced DNA fragmentation in rat thymocytes and spleen cells. Higher concentrations or prolonged exposure of organotins induced necrosis instead of apoptosis, and this could explain why until now the potency of DBTC to stimulate apoptosis *in vitro* was resulted unnoticed (only 1-6 h of incubation has been tested) (Aw *et al.,* 1990; Raffray and Cohen, 1993). Thus, apoptosis appears to be not responsible for organotin-induced thymus atrophy at a dose that inhibits immature thymocyte proliferation, although it might be involved at high doses of the compounds (Raffray and Cohen, 1993; see also appendix).

Because of the cytotoxic effects of DBTC or TBTC (see chapter 1), we aimed to further investigate the mechanisms underlying *in vitro* organotin-induced DNA fragmentation.

At lower concentrations than required to induce apoptosis, both compounds were capable of inhibiting synthesis of protein and DNA, and triggering *de novo* synthesis of heat shock proteins (HSPs). The possible relation between organotin-induced apoptosis and macromolecular synthesis has been investigated in chapter 3, with a particular interest on

DBTC that is considered the most potent thymotoxic compound (Snoeij *et al*., 1988). We have demonstrated that DBTC increased the synthesis of RNA in small high-density thymocytes ($CD4^+CD8^+$), like already shown for TBTC (Snoeij *et al.,* 1986). Results indicated that organotin-induced apoptosis affected the same subset of small thymocytes $CD4^+CD8^+$ and furthermore, that the process was RNA- and protein-synthesis dependent. Notably, the combination of the RNA and protein synthesis inhibitors (actinomycin D and cycloheximide, respectively) with DBTC or TBTC decreased the amount of DNA fragmentation induced by organotin compounds alone.

The need of *de novo* protein synthesis during apoptosis was somewhat surprising, considering that both organotins are potent protein inhibitors.

Organotin compounds induced synthesis of the heat shock protein HSC73, at concentrations that inhibited protein synthesis to around 20%. Heat shock (HS) by itself, however, was not able to increase apoptosis in rat thymocytes, contrasting previous results which indicated that HS could induce DNA fragmentation in mouse thymocytes, probably because of differences in strain-specificity (Migliorati *et al*., 1992). Because cyclohexi-mide, which by itself did not increase apoptosis, also induced heat shock proteins (HSPs), we concluded that the production of HSC73 by organotins was not due to a direct effect of the compounds, but more to a consequence of the inhibition of protein synthesis.

So, as we found that DBTC and TBTC increased synthesis of RNA, we aimed at identyfing the newly synthesized RNA molecules. In chapter 4, we therefore used the cDNA macro-array technique, which allows the analysis of multiple genes apoptosis-related simultaneously, to assess whether and if so which genes are translated after short exposure of rat thymocytes to DBTC. Two genes, glutathione S-transferase (GST) and *nur77*, were found to be activated under influence of apoptotic doses of DBTC, and both of them are known to be involved in the apoptotic process by various other chemicals (Flomerfelt *et al*., 1993; Winoto, 1994). GST, an antioxidant defence enzyme involved in cellular detoxification (Mannervik and Danielson, 1988), was proposed to be regulated by ROS (Rushmore *et al*., 1991). It seems that an increase of GST-level in apoptotic cells could be related to an enhanced production of ROS, indicating a situation of oxidative stress rather than a required step in the apoptotic pathway (already shown in case of steroid-induced apoptosis, by Flomerfelt *et al*., 1993).

Nur77 is a member of the steroid/thyroid hormone receptor superfamily, previously shown to be expressed during TCR-mediated apoptosis (Liu *et al*., 1994; Winoto, 1994), regulated by calcium signals (Woronicz *et al.,* 1995). We found that blocking the transcription of *nur77* effectively decreased the percentage of apoptotic nuclei by DBTC, confirming that *nur77* is responsible for the activation of the apoptotic process induced by organotins.

Besides effects on macromolecular synthesis, DBTC and TBTC are known to disrupt energy metabolism (Cain *et al.,* 1977; Snoeij *et al.,* 1986). TBTC has been observed also to rapidly increase the level of intracellular calcium, which is linked to a subsequent endonuclease activation and DNA fragmentation (McConkey *et al*., 1989; Chow *et al.,* 1992). At mitochondria level, TBTC caused formation of reactive oxygen species (ROS), release of proapoptotic factors, such as cytochrome *c*, and activation of caspases (Corsini *et al.,* 1996; Stridh *et al*., 1998). In chapter 5 we demonstrated the similarity between the organotin compounds DBTC and TBTC in the pathway followed to initiate apoptosis. An initial increase of calcium, followed by ROS production and release of cytochrome *c* from the mitochondrial membrane into the cytosol was also evident in case of DBTC**.** Also caspase 3 was found to be activated by DBTC**,** resulting in DNA fragmentation and cell death. ROS generation, cytochrome *c* release, and DNA fragmentation were reduced when organotin compounds were used in combination with the calcium chelator BAPTA, or with the electron transport chain-inhibitor rotenone. This indicates the important role of intracellular calcium and mitochondria in organotin-induced apoptosis.

It can be hypothesized that the cellular membrane is the first target of organotins. At relatively low concentrations/doses this leads to a proliferative arrest due to disturbance in metabolism, ATP reduction, protein and DNA synthesis inhibition. At higher concentrations/doses, a series of events, partly correlated, initiate the apoptotic process (fig. 1). The increase of calcium is one of the earliest steps, which might regulate the activation of the DNA binding transcription factor *nur77* as well, and it is followed by ROS and consequent GST production.

An increase of the permeability of the inner mitochondrial membrane (permeability transition, PT) is linked to the dissipation of the inner transmembrane potential ($\Delta\Psi$) and to

the release of the pro-apoptotic factor cytochrome *c* to the cytosol. Cytochrome *c* activates caspase 3, which cleaves specific substrates contributing to the process of apoptotic cell death.

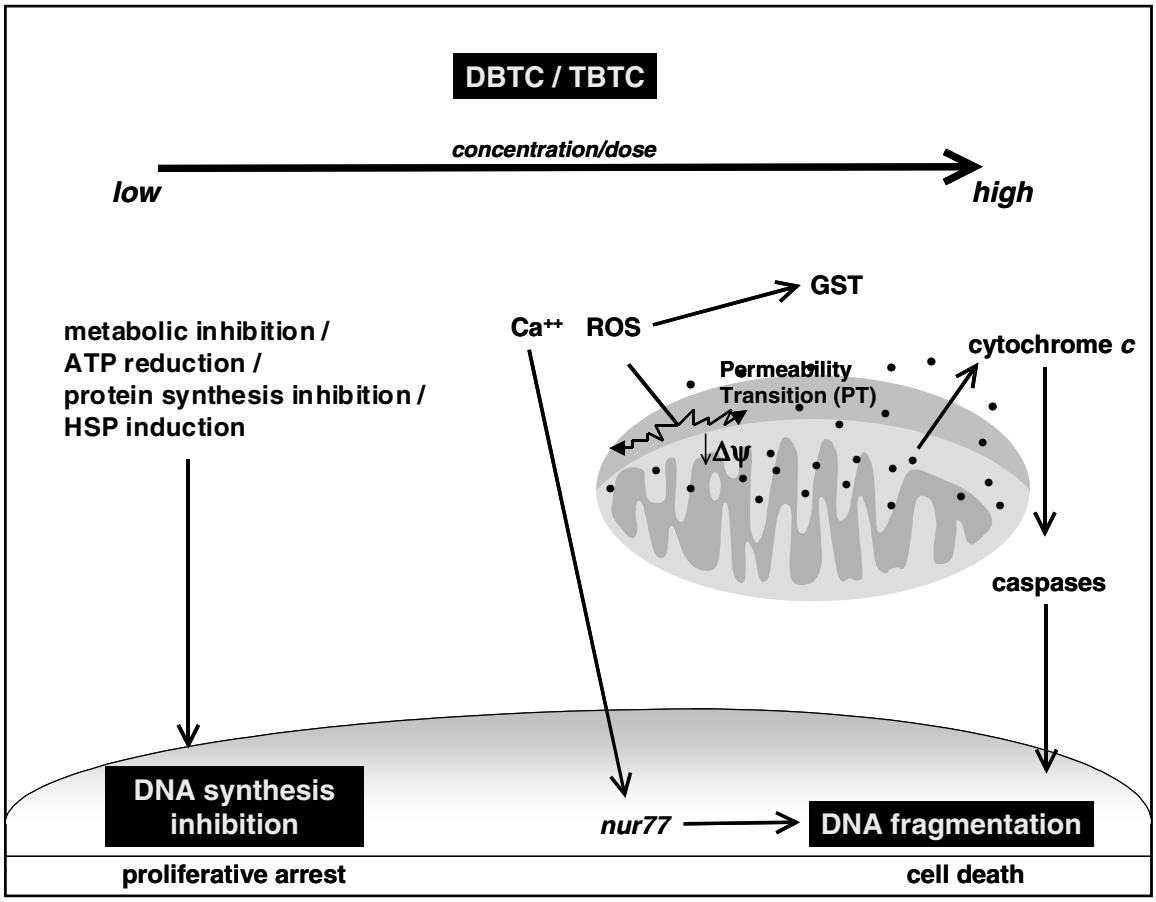

Fig. 1. Schematic representation of the events involved in proliferative inhibition and apoptosis induced by organotin compounds. Δψ: mitochondrial membrane potential. Dots indicate the cytochrome *c* molecule. From low to high concentrations means: from 0.1 μM to 3-5 μM. From low to high doses means: from 15 mg/kg to 60 mg/kg.

It is not possible to say which one of these above mentioned events is the crucial one in organotin-induced apoptosis. Each of them is involved in the initiation of the apoptotic process, and the inhibition of one single step can reduce the extent of DNA fragmentation

but not completely prevent the process. It means that the events, although linked to each other, can independently be activated and bring the cell to an irreversible damage.

In conclusion, DBTC- or TBTC-induced apoptosis is a mechanism of cell death relevant both *in vitro* and *in vivo* at relatively high concentrations/doses of compounds, while lower concentrations/doses of organotins result in anti-proliferative effects without the involvement of the apoptotic process.

Previously, it has been shown *in vitro* that DBTC is able to inhibit not only the proliferation of thymocytes, but also of T and B splenic cells (Seinen *et al*., 1979). Moreover, in the second chapter of this thesis (Gennari *et al*., 1997), it is shown that organotin compounds induce apoptosis in rat splenocytes at the same concentrations as in case of thymocytes. In the last chapter, we tested whether DBTC, *in vivo*, had a direct effect on peripheral T cell responsiveness. Interestingly, we found that DBTC could directly and rapidly (in 3 days) inhibit a hapten (DNCB)-specific immune response, reducing the number of peripheral cells and antibody forming cells (IgG) isolated from auricular lymph nodes of sensitized animals (chapter 6).

This finding has important implications for studying mechanisms of action of organotins, as it means that focus no longer has to be on inhibition of immature thymocyte proliferation but on lymphocyte proliferation in general.

In conclusion, the results described in this thesis confirm that DBTC and TBTC induce apoptosis in vitro, at relatively high concentrations. For both compounds, the apoptotic mechanism is dependent on RNA and protein synthesis (two new molecules up-regulated by organotins have been identified) and is initiated at mitochondria level, with the involvement of calcium, ROS, cytochrome c and caspases.

In vivo data indicate that apoptosis is not evident at a dose of organotins known to inhibit thymocyte proliferation.

Finally, DBTC has been found to be not thymus-selective, but to have a direct inhibitory effect on the peripheral immune responsiveness.

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APPENDIX

Re: Organotin-induced apoptosis as observed *in vitro* is not relevant for induction of thymus atrophy at antiproliferative doses (article n. TO978265)

To the editor:

These authors refer to findings previously published by us (Raffray and Cohen, 1993) on thymic atrophy caused *in vivo* by tributyltin (TBT), and we would like to offer the following comments:

1. The two studies should be considered in terms of molar dose of TBT. Their selection of a single dose level of 20 mg kg^{-1} tri-*n*-butyltin chloride (TBTC) po represents 55 µmol kg^{-1} TBT. Our study was conducted with bis(tri-*n*-butyltin)oxide (TBTO), not with TBTC as they stated, at po doses of 30 or 60 mg kg^{-1}, which represent 98 or 196 µmol kg^{-1} TBT, respectively. This difference alone significantly complicates comparison of effect between the two studies.
2. Assessment of thymic cell counts from both studies indicates clear differences in the respective kinetics of thymocyte depletion:

			Percentage of control at the indicated time point			
		Treatment	18 h	24 h	36 h	48 h
TBTC	20 mg kg^{-1}	(Gennari *et al.,* 1997)	—	101	—	58
TBTO	60 mg kg^{-1}	(Raffray and Cohen, 1993)	75	—	54	36

Thus the immunohistochemical and flow cytometric assay evaluations of apoptosis incidence shown in their paper were conducted at a time point (24 h) at which no decrease in total thymocyte count had yet occurred. They comment upon a similar absence of effect after 48 h, but no results are presented.

3. Their paper emphasizes the absence of free apoptotic cells in thymic tissue sections from animals dosed with TBT. This was also the case in our work with TBT, which was clearly stated by us, and is not in dispute. However, they did not address our comparative cytopathology findings which demonstrated treatment-related increases in phagocytes with included apoptotic thymocytes. Increased macrophage clearance activity in the thymus following triorganotin administration to rodents (at equivalent doses) has been independently noted by two other laboratories (De Waal *et al.*, 1993; Kempson *et al*., 1993).

4. Although they contend that, *in vivo*, di-*n*-butyltin (DBT) from metabolic dealkylation of TBT rather than TBT itself is the toxicologically active agent, it is highly unlikely that all TBT is dealkylated prior to significant exposure of thymus to potentially cytotoxic concentrations of TBT (see toxicokinetics data in Humpel *et al.,* 1986; and also Snoeij, 1987). In this context, even their own *in vitro* work with a brief (10 min) exposure of thymocytes to TBT, followed by washout, shows significant stimulation of apoptosis is possible at concentrations relevant to blood concentrations *in vivo.*

5. A deficiency in our study was the absence of stress-glucocorticoid measurements (this parameter was also not assessed in their study). However, certain pieces of indirect evidence we referred to, and also comparative literature data, indicate that stress-glucocorticoid-induced thymocyte apoptosis is unlikely to have been a significant feature.

6. In conclusion, it is undisputed that antiproliferative mechanisms are important in organotin thymotoxicity. However, TBT and also DBT are now known to be very cytotoxic. The title of our 1993 paper proposed consideration of apoptosis as "*a* mechanism"in triorganotin thymotoxicity, not as " *the* mechanism,"and we did not rule out concurrent antiproliferative effects. Based on the available data set in the literature, it is our opinion that apoptotic deletion of thymocyte can contribute to triorganotin thymotoxicity *in vivo*, and that such an effect will be increasingly relevant at moderate to high organotin doses. In such models the relative contribution of antiproliferative and antidevelopmental effects on thymocytes versus cytotoxicity remains to be established in quantitative terms. There is often a natural tendency to oversimplify the behaviour of experimental systems in the search for a single unifying mechanism, but this may be a misleading approach in the case of triorganotin thymotoxicity. A plausible working

hypothesis is that a composite mechanism is involved in cortical thymocyte depletion, involving complex antiproliferative or antidevelopmental effects and also cytotoxicity resulting in the activation of apoptosis.

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MARK RAFFRAY
GERALD M. COHEN

Reply

To the editor:

The aim of our study was to examine whether an increase of apoptosis occurred at doses of organotin (tributyltinchloride, TBTC, or dibutyltinchloride, DBTC) that were previously found to be selectively inhibit immature thymocyte proliferation

(demonstrated previously by decrease of DNA synthesis and number of thymoblasts). Because this inhibition of proliferation was maximal 24-48 h after dosing and thereafter recovered to reach control level again on days 4-5 after dosing, we focused on these mechanistically important early time points.

The main conclusion that can be drawn from our results is that at relatively low, antiproliferative doses of DBTC or TBTC there is no increase in apoptotic thymocytes on 24 or 48 h after dosing. By hypothesizing that low doses of these compounds have a mere antiproliferative effect (on immature $CD4^-CD8^+CD3^-$ thymocytes) all ensuing phenotypical and kinetic intrathymic changes can be explained. We fully agree with Raffray and Cohen that at higher doses of either of these organotin compounds, apoptosis and eventually also necrotic cell death may become of increasing importance and eventually the major mechanism involved. This dose dependency of the *in vivo* effects is reflected in *in vitro* experiments showing that inhibition of DNA synthesis is affected by concentrations of TBTC or DBTC that are 10 or 30 times as low as those that induce DNA fragmentation.

But although apoptosis is not unlikely to be involved at high doses, it is unclear from data of Raffray and Cohen (1993) whether they found an increase of apoptosis on a whole organ base. They show an increase in DNA fragmentation from ±10 μg DNA fragments/10^8 thymocytes in controls to 45 μg of DNA fragments/10^8 thymocytes in the 60 mg/kg group, but thymi of this latter group harbor only of 30% of the control thymocyte number: thus on a whole organ base there seems only a minor increase from approximately 10 to 15 μg of DNA fragments/thymus. Although the blood level of glucocorticosteroids is not elevated, this slight increase in DNA fragmentation might be explained by moderate stress, that may also underlie the slight loss in body weight at higher doses (30-60 mg TBTO/kg, Raffray and Cohen, 1993; 35 mg TBTC/kg, Snoeij *et al.*, 1985). If apoptosis would occur at the high doses, then it is also not surprising that macrophages appear in the thymus to clear cell remnants (Raffray and Cohen, 1993); however, at antiproliferative doses of DBTC we did not observe an increase in the absolute number of $ED2^+$ - or $ED1^+$ -cortical macrophages (Pieters *et al.*, 1988).

It might seem logical to compare TBTC and TBTO (containing two TBT groups attached by oxygen) based on the molals of the bioactive TBT+ they release. If true, it

would suffice to use half the dose (in mg/kg body wt) of TBTO to obtain the same effect as in the case of TBTC, implying that Raffray and Cohen used a much higher dose then we did (compare our 55 μmol/kg with their 195 μmol/kg). But both LD50 doses (122 mg TBTC/kg and 127 mg/kg TBTO, Schweinfurth, 1985) as well as thymotoxic doses (reduction of thymus weight: 56 and 43% at 25 mg/kg TBTO and TBTC respectively, Bressa *et al.*, 1991) are similar on a mg/kg base and also ED50 concentrations that inhibit DNA synthesis *in vitro* are comparable (compare Snoeij *et al.,* 1985, with Vos *et al.,* 1984). Apparently, hydrolysis of TBTO to 2x TBTOH and subsequent reaction to TBT+ salt (Bock *et al.*, 1962) is less efficient than dechlorination of TBTC to TBT+. This would mean that the TBTO and TBTC are more comparable at the mg/kg than at the molar base.

Thymic effects of TBTC and DBTC upon single *iv* treatment

Compound	Dose (mg/kg)	*n*	Body wt (g)	Rel. thymus wt (mg/g)
	0	5	77.3±4.6	3.73±0.73
	0	15	76.5±3.8	3.67±0.61
DBTC	0.5	5	78.7±5.6	3.36±0.61
	1.0	5	76.8±2.0	2.74±0.47*
	2.5	5	78.9±3.7	2.48±0.41**
TBTC	0.5	5	79.1±3.8	3.47±0.38
	1	5	76.5±4.6	3.74±0.42
	2.5	5	78.1±2.1	3.70±0.46
	2.5	15	78.7±3.8	3.40±0.51

Note. Mean ± SD, *p<0.01, **p<0.001.

Finally, we fully agree with Raffray and Cohen that debutylation of TBT+ to DBT+ is incomplete, and much TBT+ appears in the circulation after oral exposure to TBTC (Snoeij, 1987). Most data that support the idea that TBT must be converted to DBT to become biologically active are given in Snoeij *et al.*, (1988). Further support for the idea that TBTC itself is not directly thymotoxic and must be metabolized (to DBT+) via the

oral route comes from an additional series of experiments, that is not published in international literature (Snoeij, 1987) and given in the table. It shows that intraveneous treatment with DBTC but not with TBTC causes thymus atrophy. Thus, despite the fact that the blood concentration of TBTC is high, no thymic effects are induced, whereas DBTC is very potent in inducing thymus atrophy via the intraveneous route.

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ALESSANDRA GENNARI
RAYMOND PIETERS

SAMENVATTING IN HET NEDERLANDS

Organotinverbindingen vormen een groep van stoffen die een breed scala aan toepassingen kent en in volume de meest geproduceerde organometaalverbindingen zijn. De bekendste van deze verbindingen zijn de organotinverbindingen waarbij aan een tin-atoom drie aryl- of alkyl-groepen gesubstitueerd zijn. Sommige van deze trialkyltinverbindingen worden gebruikt als schimmeldodend middel (fungicide) in de aardappelteelt, anderen zijn vooral bekend als bestanddeel van aangroeiwerende verven op scheepshuiden. Een probleem bij deze toepassing is dat de stoffen uit de verven lekken en in het water terecht komen. Vanwege de hormoonverstorende werking van trialkyltinverbindingen bij zoutwaterslakken worden deze stoffen in verband gebracht met de achteruitgang in de populatie purperslakken en wulken in onze kustwateren.

Een minder bekende klasse van organotinverbindingen wordt gevormd door stoffen waarbij slechts twee alkylgroepen aan het tin-atoom vastzitten. Deze dialkyltinverbindingen worden gebruikt als katalysatoren in de polyurethaansynthese (PUR-schuim) en als stabilizatoren in PVC plastics. De organotinverbindingen die in dit proefschrift gebruikt zijn, zijn tributyltinchloride (TBTC) en dibutyltin dichloride (DBTC).

Door de brede toepassingsmogelijkheden is het gebruik en produktie van deze stoffen sinds hun ontwikkeling halverwege de vorige eeuw enorm toegenomen. Deze toename heeft er ook toe geleid dat de giftigheid van deze stoffen grondig onderzocht is. Maar pas rond 1976 werd uit proefdierexperimenten bekend dat met name het afweersysteem gevoelig is voor dibutyltinverbindingen, hetgeen later ook het geval bleek voor tributyltinverbindingen. Het meest opvallende effect van de verbindingen is een verandering in de thymus, beter bekend als de zwezerik. De verbindingen zorgen namelijk voor een verschrompeling van de thymus, een effect dat ook wel aangeduid wordt als thymusatrofie.

De thymus vervult in het afweer of immuunsysteem een belangrijke, centrale functie. In dit orgaan vindt namelijk de ontwikkeling plaats van T (Thymus-afhankelijke) cellen.

Deze cellen vervullen een dirigerende rol in de afweerreactie tegen bijvoorbeeld bacteriën, virussen en tumoren. Tijdens de ontwikkeling in de thymus leren onrijpe pre-T cellen, die afkomstig zijn uit het beenmerg, waartegen ze wel en waartegen ze niet mogen reageren. Tijdens deze, bijzonder ingewikkelde, T-celontwikkeling spelen tal van processen een rol. In eerste instantie vindt er een sterke vermeerdering van pre-T cellen plaats. Tijdens deze vermeerdering verschijnen er op de buitenkant van de cellen zeer specifieke receptoren, T cell receptoren, die fragmenten van eiwitten kunnen herkennen. Vervolgens wordt, nog in de thymus, nagegaan of de T cellen daadwerkelijk iets kunnen herkennen. Dit is nodig om te kunnen reageren op een ongewenste indringer, zoals een bacterie. Maar ze mogen ook weer niet te heftig reageren op stukjes eiwit die van nature in ons lichaam aanwezig zijn. Dit laatste zou namelijk kunnen zorgen voor een afweerreactie tegen de eigen organen of weefsels. Het is na vele jaren van onderzoek gebleken dat T cellen die helemaal niet reageren en ook T cellen die te sterk reageren in de thymus afsterven. Het stervensproces is een nauwkeurig gecontroleerd proces dat apoptose genoemd wordt. Bij apoptose wordt de cel zodanig opgeruimd dat dit geen overmatig celdebris, met als mogelijk gevolg ontstekingen, oplevert. Kenmerkend voor dit proces is dat het DNA netjes afgebroken wordt en dat het afhankelijk is van de metabole aktiviteit van de cel.

Sinds de ontdekking dat tri- en dibutyltinverbindingen een effect op de thymus hebben is veel onderzoek gericht op de gevolgen van het effect voor de afweer. Bovendien is er veel energie gestopt in het ophelderen van het werkingsmechanisme van deze stoffen, met andere woorden in het karakteriseren van de processen waarvan de verstoring cruciaal is voor het gevonden effect.

Uit het onderzoek bleek al snel dat het effect op de thymus uiteindelijk leidt tot een afname van T cellen in de rest van het lichaam, vooral in de milt en de lymfknopen. Deze afname heeft op haar beurt gevolgen voor het functioneren van de afweerreactie, met name de T cel-afhankelijke afweerreacties. Ook is al vroegtijdig gebleken dat met name TBTC en DBTC behoorlijk giftig zijn voor T cellen als ze in een kweekbuis direct in contact gebracht worden met de stoffen. Bij lage concentraties wordt met name de celdeling van T cellen geremd. Hoe dit precies in zijn werk gaat is nog steeds onduidelijk maar

vermoedelijk grijpen deze stoffen tegelijkertijd aan op meerdere processen in de cel, waardoor een verstoring van de energiehuishouding en uiteindelijk een remming van energievragende processen optreedt. Ook in ratten die aan lage doseringen werden blootgesteld is een afname van de celdeling in de thymus gevonden. Bij een eenmalige blootstelling blijft dit effect beperkt tot de thymus, hetgeen heeft geleid tot de conclusie dat deze stoffen thymus-selectief werken.

Recenter is gebleken, met name in geval van de trialkyltinverbindingen, dat hogere concentraties celdood, door middel van apoptose, tot gevolg hebben. In een vervolg op deze reageerbuis-experimenten werd aangetoond dat hoge doseringen trialkyltinverbindingen ook apoptose in de thymus van ratten kunnen veroorzaken. Deze bevinding was deels in tegenspraak met de eerdere ideëen dat organotinverbindingen met name de celdeling verstoorde en bovendien met het vermoeden dat trialkyltinverbindingen omgezet worden in dialkyltinverbindingen en dat deze laatste verbindingen het uiteindelijke effect hebben.

Het eerste **doel van het onderzoek** beschreven in dit proefschrift was dan ook om na te gaan of DBTC, net als TBTC, apoptose kan veroorzaken in de thymus van de rat. En indien dat zo zou zijn, na te gaan welke biochemische processen bij dit proces van apoptose een rol spelen.

Een **ander belangrijk doel** van dit onderzoek was om na te gaan of deze stoffen, met name DBTC, alleen de celdeling in de thymus verstoort. Dit was belangrijk omdat jarenlang onderzoek, beschreven in een viertal proefschriften, nog steeds niet had uitgewezen waarom nou juist celdeling in de thymus gevoelig is voor DBTC. Hierbij dient nog opgemerkt te worden dat in de reageerbuis zowel de deling van T cellen uit de thymus als van T cellen uit de milt geremd wordt door DBTC.

Het onderzoek beschreven in hoofdstuk 2 van dit proefschrift toont aan dat apoptose niet optreedt bij doseringen (15-20 mg/kg lichaamsgewicht) die al wel de celdeling remmen in de thymus. Bovendien wordt in dat hoofdstuk aangetoond dat DBTC in de reageerbuis ook, net als TBTC, in staat is apoptose te veroorzaken, en niet alleen in T cellen uit de thymus maar ook in T cellen uit de milt (!)

In hoofdstuk 3 wordt het proces van apoptose als gevolg van DBTC nader bestudeerd. Hieruit blijkt dat met name een speciaal type van T cellen uit de thymus, namelijk de onrijpe en apoptose gevoelige dubbelpositieve cellen (deze cellen hebben zowel een CD4 als een CD8 receptor), gevoelig zijn voor celdoding door DBTC. Tijdens dit proces vindt er in deze cellen een nieuwvorming plaats van RNA-moleculen en bepaalde eiwitten die heat-shock eiwitten genoemd worden. Remming van de RNA synthese of de eiwitsynthese verhindert de apoptose als gevolg van DBTC hetgeen aangeeft dat de nieuwvorming essentieel is voor het optreden van apoptose. Onduidelijk blijft in dit hoofdstuk voor welk eiwit deze RNA-moleculen coderen. Wel is duidelijk dat dit waarschijnlijk niet de heat-shock eiwitten.

De verdere karakterisatie van de nieuwgevormde RNA-moleculen is gedaan in hoofdstuk 4 met behulp van de cDNA macro-array techniek. Twee genen, één coderend voor glutathione S-transferase (GST) en één voor *nur77*, bleken toegenomen in T cellen uit de thymus na kortdurende blootstelling aan DBTC. GST is betrokken bij de ontgiftiging of detoxificatie-mechanismen van cellen en wordt gereguleerd door reactieve zuurstofmoleculen die in een vergiftigde cel kunnen ontstaan. *Nur77* is een lid van een zogenaamde superfamilie van hormoonreceptoren, de steroid/thyroid hormone receptor superfamilie, en is betrokken bij apoptose in thymale T cellen. Aktivatie van *nur 77* wordt geregeld door calcium signalen. Bijzonder interessant is dat door de transcriptie van *nur77* te blokkeren door middel van een stukje complementair DNA, de apoptose als gevolg van DBTC voor een aanzienlijk deel geremd wordt. Dit toont aan dat *nur77* inderdaad een rol speelt in het apoptose-proces dat door DBTC geaktiveerd wordt.

Het mogelijke verband tussen verstoorde cellulaire processen en apoptose wordt bestudeerd in hoofdstuk 5. Hierin wordt beschreven dat TBTC en DBTC via een vergelijkbare verstoring van processen apoptose veroorzaken. Beide stoffen blijken in staat om de concentratie van calcium te verhogen, de produktie van reactieve zuurstofmoleculen te induceren, te zorgen voor het vrijkomen van cytochroom *c* uit mitochondria in het cytosol en caspase 3 te aktiveren. Al deze processen kunnen in meer of mindere mate bijdragen aan het proces van apoptose, hetgeen blijkt uit studies waar calcium specifiek wordt weggevangen of waarbij de vorming van reactieve zuurstof

radicalen geremd wordt. Ook onderdrukking van caspase 3 werking remt de apoptose als gevolg van de DBTC.

In hoofdstuk 6 wordt in een proefdiermodel nagegaan of DBTC ook een effect heeft op T cel-deling buiten de thymus. In dit model wordt een immuunrespons opgewekt, door een immunostimulerende allergene verbinding (DNCB) op drie achtereenvolgende dagen op het oor van een rat aan te brengen. De immuunreactie is na een week afgelezen aan de hand van zwelling (celaantal) van de lymfknoop die dicht bij het oor gelegen is. Bovendien is de immunologische aktiviteit (antilichaamreactie tegen de verbinding DNCB) in de knoop bepaald. DBTC werd aan dezelfde rat gegeven op het moment dat de celdeling in de knoop maximaal was (4 dagen na de eerste keer opbrengen). Uit de resultaten blijkt dat DBTC wel degelijk in staat is ook de celdeling te remmen van T cellen in de lymfknoop, dus buiten de thymus. Dit is nooit eerder opgemerkt, waarschijnlijk omdat altijd gekeken is in ratten die immunologisch in rust waren of in dieren die al langere tijd blootgesteld waren aan organotinverbindingen zodat elk effect buiten de thymus verdoezeld werd door de gevolgen van de thymusatrofie, namelijk een verminderde uitstroom van uitgerijpte T cellen. De nieuwe bevinding kan een duidelijke invloed hebben op eventueel verder onderzoek naar de werkingsmechanismen van organotinverbindingen omdat de te onderzoeken processen betrokken bij celdeling of proliferatie nu niet meer thymus-specifiek hoeven te zijn.

Wat het werkingsmechanisme van organotinverbindingen betreft is er met dit proefschrift een duidelijke stap voorwaarts gezet: lage concentraties of doseringen remmen de celdeling, hoge concentraties of doseringen induceren apoptose. In beide gevallen is een afname van T cellen in de thymus of daarbuiten het gevolg. Onduidelijk en waard te onderzoeken blijft of vergelijkbare celbiologische en biochemische processen betrokken zijn bij zowel remming van celdeling als inductie van apoptose door organotinverbindingen.

RIASSUNTO IN ITALIANO

Gli organostannici sono composti chimici utilizzati in svariati settori, ad esempio in campo industriale come catalizzatori e stabilizzatori o in agricoltura come pesticidi. L'interesse nei confronti dei dialchil- e trialchil-derivati (tra cui il DBTC ed il TBTC) è dovuto alla loro azione immunotossica nei roditori. Poichè il primo bersaglio di questi composti è il timo, lo scopo di questa tesi è stato quello di esaminare i meccanismi cellulari e molecolari responsabili dell'atrofia timica, con un particolare interesse al possibile coinvolgimento dell'apoptosi nella morte cellulare.

Mentre *in vitro* gli organostannici hanno un effetto antiproliferativo su diversi sistemi cellulari, *in vivo* mostrano una selettività verso il timo, dove provocano una riduzione del peso e della cellularità. Nel secondo capitolo della tesi, in cui abbiamo condotto studi per esaminare se l'apoptosi potesse essere coinvolta in questa atrofia timica, non sono state osservate cellule apoptotiche nel timo di animali esposti a dosi di DBTC o TBTC (15-20 mg/kg) in grado di inibire la proliferazione di timociti. Gli studi *in vitro* hanno evidenziato in timociti e splenociti di ratto incubati per breve tempo (10 min) a concentrazioni relativamente alte (3-5 μM) di stanno-derivati, la frammentazione del DNA e quindi la capacità di questi composti di indurre apoptosi.

Poichè il DBTC ed il TBTC rappresentano dei potenziali modelli di studio per esaminare i meccanismi d'azione dei composti chimici in grado di provocare apoptosi, abbiamo deciso di approfondire la nostra ricerca *in vitro* sui meccanismi molecolari alla base dell'apoptosi indotta da organostannici nei timociti di ratto.

E' noto che a concentrazioni minori di quelle richieste per indurre l'apoptosi, gli stanno-derivati inibiscano la sintesi proteica e di DNA ed aumentino la sintesi di RNA e di heat shock proteins (HSPs). Nel terzo capitolo, abbiamo dimostrato che il DBTC (in maniera simile al TBTC) stimola la sintesi di RNA nei timociti di classe $CD4^+CD8^+$, che comprende lo stesso gruppo di cellule sensibili all'apoptosi. Inoltre abbiamo dimostrato che il processo apoptotico è dipendente dalla produzione di RNA e di proteine, in quanto l'aggiunta degli inibitori di sintesi riduce l'estensione della frammentazione del DNA indotta sia dal DBTC che dal TBTC. E' interessante notare che nonostante gli stanno-derivati inibiscano la sintesi proteica, siano in grado di indurre la proteina heat-shock

HSC73. Questa apparente discordanza, può essere spiegata considerando che le HSPs sono prodotte in seguito ad uno stress cellulare, come meccanismo di difesa, quindi la produzione di HSC73 non è dovuta ad un effetto diretto degli stanno-derivati, ma piuttosto è una conseguenza dell'inibizione della sintesi proteica.

Poichè sia il DBTC che il TBTC stimolano la sintesi di RNA, nel quarto capitolo abbiamo utilizzato una tecnica moderna (cDNA macro array) per identificare i geni attivati in seguito ad una breve esposizione di timociti al DBTC. Sono stati trovati due geni, la glutatione S-trasferasi (GST) ed il *nur77*, entrambi coinvolti nel fenomeno apoptotico indotto da diversi stimoli. La GST è un enzima di difesa, prodotto durante i processi di detossificazione cellulare, mentre il *nur77* è un membro della superfamiglia dei recettori steroidei/tiroidei. Inibendo la trascrizione del *nur77*, la percentuale dei nuclei apoptotici indotti dal DBTC è risultata notevolemente ridotta, confermando l'importanza di questo gene nell'attivazione del processo apoptotico da parte degli stanno-derivati.

Oltre ai citati effetti sulla sintesi di macromolecole, il DBTC ed il TBTC sono in grado di interferire con il metabolismo energetico e con i mitocondri. Nel quinto capitolo abbiamo descritto il percorso, rivelatosi simile sia per il DBTC che per il TBTC, per indurre l'apoptosi. In particolare, sono stati osservati un iniziale aumento di calcio intracellulare, seguito dalla produzione di specie radicaliche a livello mitocondriale, dal rilascio del citocromo *c* dalla membrana mitocondriale al citosol, e dall'attivazione della caspasi 3 che porta la cellula alla frammentazione del DNA. Il fondamentale ruolo del calcio e dei mitocondri nel processo apoptotico, è stato confermato dalla riduzione della percentuale di apoptosi indotta dagli stanno-derivati in caso di aggiunta di chelatori del calcio o di inibitori della catena di trasporto di elettroni.

Possiamo quindi affermare che l'apoptosi sia rilevante ad alte concentrazioni/dosi di DBTC o TBTC, mentre piu' basse concentrazioni/dosi di questi composti interferiscono con la proliferazione cellulare senza attivare il fenomeno apoptotico.

Basandosi sul fatto che dati in letteratura riconoscono la capacità del DBTC di inibire *in vitro* non solo i timociti ma anche le cellule spleniche, nell'ultimo capitolo abbiamo esaminato se *in vivo* il composto fosse effettivamente selettivo verso il timo o avesse un generale effetto immunosoppressivo. I risultati hanno rivelato che il DBTC direttamente e rapidamente riduce il numero di cellule periferiche e di cellule formanti gli anticorpi

(IgG) nei linfonodi auricolari di ratti precedentemente immuno-stimolati, indicando che il timo non è l'unico bersaglio degli organostannici.

In conclusione, affermiamo che concentrazioni relativamente elevate degli stanno-derivati DBTC e TBTC inducono apoptosi in vitro. Il processo apoptotico è dipendente dalla sintesi di RNA e proteine ed inizia con un aumento di calcio, seguito dalla produzione a livello mitocondriale di specie radicaliche, dal rilascio del citocromo c e dall'attivazione delle caspasi. Inoltre, sono stati identificati due geni trascritti dopo l'esposizione di timociti al DBTC e coinvolti nel processo apoptotico.

Studi in vivo rivelano che l'apoptosi non sia evidente a dosi di stanno-derivati in grado di inibire la proliferazione di timociti.

Infine, il DBTC non è un composto selettivo per il timo, ma è in generale immunosoppressivo.

CURRICULUM VITAE

Alessandra Gennari is born on April 13, 1968 in Turin, Italy. In 1987, she graduated at the Liceo Scientifico Statale P. Gobetti, Turin and in 1993 she took a degree in Biological Sciences at the University of Turin. In 1995 she started a two-year EC fellowship (Human Capital & Mobility) at the University of Utrecht, The Netherlands, RITOX, Department of Immunotoxicology, under the supervision of Dr. Pieters. After going back to Italy, she worked for one year as a biologist in a private hospital and as a sales representative for scientific equipment in Turin. Then, she went back to Utrecht, RITOX, for another two-year period to obtain a PhD. She spent one year of her PhD as a guest at the University of Milan, Italy, Department of Pharmacological Sciences, under the supervision of Dr. Corsini.

In November 2000 she will defend her thesis in Utrecht.

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