Lymphocytotoxicity and Immunosuppression by Organotin Compounds. Suppression of Graft-versus-Host Reactivity, Blast Transformation, and E-rosette Formation by Di-*n*-Butyltindichloride and Di-*n*-Octyltindichloride.

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Abstract: Di-*n*-butyltindichloride (DBTC) and di-*n*-octyltindichloride (DOTC) represent a new group of organometallic compounds with antilymphocytic properties. In rats they induce lymphocyte depletion in thymus and thymus-dependent areas of spleen and peripheral lymph nodes without signs of myelotoxicity or a generalized toxicity. The number and viability of cells isolated from thymus and peripheral lymphoid organs was severely decreased, whereas the number and viability of bone marrow cells was not reduced. Immunosuppressive properties of DBTC and DOTC are indicated, in this study, by a severe decrease of the graft-versus-host response and the response to the T-cell mitogens phytohemagglutinin and concanavalin A. The T-cell selectivity of these compounds is discussed. In vitro DBTC and DOTC are extremely cytotoxic. Blast transformation of human as well as rat thymocytes was already inhibited at concentrations as low as 0.02 μg DBTC (or 0.1 μg DOTC) ml medium. Also the E-rosette formation was inhibited at very low drug levels. The similarity of effects upon rat and human lymphocytes suggests that DBTC and DOTC acts in the same manner in rat and man and offers the possibility of a therapeutic use of these compounds.

Key Words: Organotin compounds; Lymphocytotoxicity; Immunosuppression

INTRODUCTION

Di-*n*-butyltindichloride and di-*n*-octyltindichloride have primarily used in industry, in particular, they act as heat stabilizers of poly (vinyl chloride) (PVC) plastics (Ross, 1965; Piver, 1973). Since these compounds induce lymphocyte depletion in thymus and thymus-dependent lymphoid areas (Seinen and Willems, 1976; Seinen et al., 1977a) they represent a new group of antilymphocytic agents. Following administration (oral and intravenous), lymphoid atrophy occurs at concentrations that do not compromise other organ systems. For example, rats fed a diet containing 50 or 150 mg DOTC/kg for 4 weeks showed a reduction in the total number of thymocytes of 33 and

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6%, respectively. The viability of these cells was also dose-relatedly decreased. Like the number and viability of thymus cells, the number and viability of spleen and peripheral lymph node cells was also decreased by DOTC, but less severely so. However, the number and viability of bone marrow cells was not affected by DOTC and DBTC treatment.

Via the spleen colony assay of Till and McCulloch (1961) effects on bone marrow stem cells were excluded (Seinen and Penninks, 1978). This indicates a selective effect of DBTC and DOTC on thymus and thymus-dependent lymphocytes, which is not mediated by an increased release of glucocorticosteroids, since thymus atrophy is also observed in adrenalectomized rats (Seinen and Willems, 1976).

Further evidence for a selective cytotoxicity of DBTC and DOTC was provided by in vitro studies (Seinen et al., 1977a). Thymocyte survival decreased in direct proportion to the organotin concentration in the nutrient medium. Thymocyte number was already decreased after incubation with a concentration as low as 0.05 μg of DBTC/ml medium for 24 hr. The thymocyte count progressively diminished to 31% and thymocyte viability to 61% of the control value after 24 hr of incubation with 50 μg DBTC/ml. In contrast to the effect of rat thymocytes, DBTC and DOTC did not reduce rat bone marrow cell survival under the same culture conditions. Number and viability of rat bone marrow cells were the same when cultured with 0, 5, or 50 μg of DBTC or DOTC/ml for periods up to 40 hr.

In rats, the immune response is distinctly disturbed by DBTC and DOTC (Seinen et al., 1977b). Suppression of cell-mediated immunity occurred in such manifestations as skin graft rejection and tuberculin hypersensitivity. Inhibition of humoral immunity is shown by the reduction of plaque-forming cell numbers and hemagglutinin titers against SRBC. However the thymus-independent humoral response on *E. coli* LPS was not affected. The blood clearance of carbon particles was not impaired by DOTC treatment. This suggests the absence of any effect on macrophages.

It was demonstrated, as this article discusses, that the graft-versus-host (GVH) response and the response to the T-cell mitogens phytohemagglutinin (PHA) and concanavalin A (Con A), hence the quantities recoverable, from organotin-treated rats were severely depressed. In vitro treatment of rat and human lymphocytes resulted in decreased cell survival, mitogen response, and E-rosette numbers even at low organotin concentrations. The recorded data indicate a similar effect of DBTC and DOTC in lymphocytes of both rat and man.

MATERIALS AND METHODS

Materials

Di-*n*-butyltindichloride (purity >98%) and di-*n*-octyltindichloride (purity >98%) were kindly provided by Dr. E. J. Bulten (Institute for Organic Chemistry TNO, Utrecht, The Netherlands).

Animals

Inbred Wistar-derived WAG rats and (WAG × B)F_1 hybrids were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. The parental strains (WAG and B) apparently differ at an important histocompatibility locus, since skin allografts in the same strains were rejected after approximately 10 days (Seinen et al., 1977b). For pre- and postnatal studies (see below), virgin females were mated and shipped at day 1 of pregnancy. At birth, the number of neonates per litter was standardized to six.

Abbreviations. GVH: graft-versus-host; PHA: phytohemagglutinin; Con A: concanavalin A; LPS: *E. coli* lipopolysacharide O 127: B 8; PLN: peripheral lymph node; DBTC: di-*n*-butyltindichloride; DOTC: di-*n*-octyltindichloride; ^{3}H-TdR: tritiated thymidine; b.w.: body weight; BM: bone marrow; SRBC: sheep red blood cells; PBS: phosphate-buffered saline.

Cell Suspensions

Rat lymphoid organs were removed under aseptic conditions (the thymus was carefully trimmed free from adjoining lymph nodes), weighed, and minced with scissors in cold RPMI 1640 medium (Gibco, Paisly, Scotland) or phosphate-buffered saline (PBS). Tissue fragments and femoral bone marrow were screened through a nylon sieve (pore diameter 225 μm). To remove cell clumps the suspensions were passed through a 25-gauge needle. The cells were counted in an electronic particle counter (Coulter Counter, model ZF, Coulter Electronics Ltd, Bedford, England) and the total number of nucleated cells per organ calculated. Cell viability was determined in chambers with 0.05% nigrosin (Merck A.G., Darmstadt, Germany) solution in saline.

Corticosteroid resistant lymphocytes were isolated from thymuses of rats pretreated 3 days earlier with a single intraperitoneal (ip) injection of 15 mg hydrocortisone acetate (Hydro-Adreson, Organon, Oss, The Netherlands).

Human thymus tissue was removed from children during thoracic surgery at the Antonius Hospital, Utrecht, The Netherlands, and obtained courtesy of Prof. Dr. H. A. Huysmans. Cell suspensions were prepared as described for rat cell suspensions.

Graft-versus-Host Assay

Thymus, spleen, and lymph node cells from WAG parent rats were injected subcutaneously (sc) into the hind feet of young adult (WAG × B)F_1 hybrid recipient animals (Ford et al., 1970). The GVH-inducing potency of cells of control and treated animals was assessed by injecting them into the right and the left feet, respectively. In pilot studies it was shown that the mean weight of the draining popliteal lymph node 7 days after injection was linearly related to the dose of thymus, spleen, and lymph node cells on a double log scale.

The slope of these lines was determined according to the curve of best fit. Assuming that the slopes of the dose–response curve of cell preparations of treated and of control groups were similar, the relative GVH-inducing potency of treated cells was calculated. From these data and the total number of viable cells per organ, the relative GVH activity on a whole organ basis was estimated.

Lymphocyte Transformation

Lymphoid cells (4×10^5 cells in 0.15 ml of medium) were cultured in the wells of U-form microtiter plates with loosely fitting lids (type M 220-24 AR, Greiner, Nürtingen, Germany). The medium consisted of bicarbonate-buffered RPMI 1640 medium supplemented with 50 U of penicillin and 50 μg of streptomycin/ml (all from Gibco-Biocult, Paisly, Scotland), 2mM L-glutamine (Schwarz/Mann, Orangeburg, N.Y.), and 15% fresh isologous rat serum or fetal calf serum (fcs) (Difco Lab., Detroit, Mich.) decomplemented at 56°C for 30 min. The plates were wrapped in plastic adhesive kitchen film to prevent evaporation in the wells at the edge of the plate (Du Bois et al., 1973) and incubated for 72 hr at 37°C in a humid atmosphere of 5% CO_2 in air. Dose–response curves for mitogens showed doses of 5 μg of PHA (PHA-P, Difco Lab., Detroit, Mich.), 2 μg Con A, crystallized twice (Nutritional Biochemicals Corp., Cleveland, Ohio), and 0.25 μg LPS (Difco Lab., Detroit, Mich.) per well to be optimal. After 48 hr of culture, 0.25 μCi tritiated thymidine (^{3}H-TdR, specific activity 0.5 Ci/mmol, The Radiochemical Centre, Amersham, England) in 20 μl medium was added. After an additional 24 hr of incubation, the cells were harvested onto glass fiber filters using a multiple cell culture harvester (Skatron, Lierbyen, Norway). Filters were dried and transferred to scintillation vials containing 5 ml of scintillation fluid (5 g 2,5-diphenyloxazole) (PPO) and 50 mg 2,2′-*p*-phenylene *bis* (5-phenyloxazole) (POPOP)) per liter reagent grade toluene (Merck, Darmstadt, Germany) and counted in a Mark II (Nuclear Chicago) scintillation counter. The mean uptake of ^{3}H-TdR in triplicate cultures was calculated and the results expressed as the differences in counts per minute (Δ cpm) between stimulated

and control cultures or as percentages of ^{3}H-TdR incorporation of the control cultures. Standard deviations of triplicate cultures never exceeded 10%.

Rosette Formation

Aliquots of 0.1 ml of a suspension containing 1×10^8 twice washed SRBC/ml were pipetted into polystyrene culture tubes (12 × 75 mm, Falcon, Oxnard, Calif.) containing 2×10^5 human thymus cells. The mixture was centrifuged at 200 × *g* for 5 min and then incubated at room temperature for 1 hr. After the supernatant was discarded, the cell pellets containing the rosettes were gently resuspended before a droplet of a 0.1% solution of toluidine blue was added. A rosette was defined as a nucleated cell having 3 or more SRBC bound to its surface. A total of 100 mononuclear cells per tube were examined and results were expressed as the mean percentage of rosette-forming cells of triplicate incubations.

Treatment of Rats with Di-*n*-Butyltindichloride or Di-*n*-Octyltindichloride

Postnatal treatment with DOTC. DOTC was dissolved in arachis oil and the rat pups were dosed by gavage according to their individual body weights. From the second day after birth, pups were dosed 3 times a week with DOTC. Litters were standardized to six pups and two animals per litter received 0, 5, or 15 mg DOTC/kg body weight. For cell culture experiments 4-week old animals were used.

Treatment of weanling rats with DBTC or DOTC. Groups of 3-week-old male weanling WAG rats of approximately 40 g received diets containing 0, 50, or 150 mg DBTC or DOTC/kg diet. Animals were killed after an experimental period of 31 days, and thymus, spleen, and peripheral (brachial, axillary, and inguinal) lymph nodes were weighed. Cell suspensions of these organs were pooled per group and used in a GVH assay.

Pre- and postnatal treatment with DOTC. From day 2 of pregnancy rats were fed diets containing 0, 50, or 150 mg DOTC/kg body weight. The pups were exposed prenatally by limited transplacental passage of DOTC and postnatally by secretion of DOTC in milk and directly from the food. Thymus and spleen of 6-week-old female rats were used for a GVH assay.

In Vitro Treatment of Rat and Human Thymocytes

Two million rat or human thymocytes were cultured, in a total volume of 0.5 ml of medium, in loosely capped polystyrene culture tubes (12 × 75 mm, Falcon, Oxnard, Calif.). Graded amounts of DBTC and DOTC, dissolved in ethanol, were added to the cultures and the total number and viability of the thymocytes were scored at different times.

To tubes containing 3×10^5 human thymocytes in 1 ml of medium, increasing amounts of DBTC and DOTC were added. The tubes were gassed with 10% CO_2 in air, tightly capped and, to prevent agglutination of the cells, incubated in a shaking water bath. At various times the number of rosette-forming cells, as well as the total number and viability of the cells, was determined.

The mitogen response of rat thymus and spleen cells and human thymocytes was studied in RPMI medium containing various amounts of DBTC or DOTC dissolved in ethanol. The ethanol concentration used (0.5%), did not reduce the responsiveness of cells to the mitogens. In some experiments, thymocyte suspensions were preincubated 4 hr at 37°C with different concentrations of DOTC or DBTC; then, after repeated washings, mitogens were added. Alternatively, graded amounts of DOTC or DBTC were added 24 hr after the 3-day culture period was initiated.

Statistical Analysis

Student's *t* test or the distribution-free Wilcoxon test were used to calculate one-sided significances of differences between test and culture values.

RESULTS

Mortality

Pre- and postnatal exposure to a 150 mg DOTC/kg diet resulted in growth stunting and mortality of 80% of the pups. They died without any major pathology except for severe atrophy of the lymphoid organs. Some animals of the 150 mg/kg group weighed 20 g when they were 1-month old, whereas control and 50 mg/kg group animals averaged 100 g. The survivors of the 150 mg/kg regimen averaged 67 g at 31 days of age. In the 50 mg/kg diet group mortality did not occur. Weight gain in this group was slightly reduced in the second month. At an age of 53 days, male rats of this group weighed 156 ± 10 g while control animals weighed 174 ± 9 g.

Number and Viability of Lymphocytes in Rats Exposed to Di-*n*-Octyltindichloride

Treatment of rats with DOTC either by gavage or diet resulted in a dose-related decrease of total cell number and viability of thymus, spleen, and peripheral lymph node cells (Table 1). The thymus was the most severely affected organ. In contrast, the number and viability of femoral BM cells was not affected. When rats were postnatally exposed to 5 or 15 mg DOTC/kg body weight for 25 days (Table 1, experiment 2), the effects on lymphoid organs were even more pronounced. Thymus contained 9 and 3%, respectively, of the number of thymocytes of control animals. Also the number of femoral BM cells of rats were decreased. The viability of these cells remained unaffected. The body weights were also dose-relatedly reduced after this treatment.

Graft-versus-Host Reactivity of Lymphocytes from Di-*n*-Butyltindichloride- or Di-*n*-Octyltindichloride-treated Rats

The GVH activities of thymus, spleen, and PLN cells of animals exposed to DBTC from weaning age for 31 days is given in Table 2 (experiment 1). On a cell for cell basis the GVH activity

Table 1 *Effect of DOTC treatment of rats on body weight and on number and viability of lymphocytes*[a]

	Total cell number ($\times 10^6$)				Viability (%)				
Doses of DOTC	*BM*	*thymus*	*spleen*	*PLN*	*BM*	*thymus*	*spleen*	*PLN*	*Body wt (g)*
mg/kg diet				EXP. 1[b]					
0	94 ± 18	1197 ± 273	539 ± 11	146 ± 20	91 ± 4	88 ± 4	81 ± 4	79 ± 10	161 ± 14
50	97 ± 17	226 ± 127[f]	410 ± 46[f]	117 ± 32	86 ± 4	59 ± 4[f]	63 ± 9[e]	59 ± 4[e]	151 ± 17
150	112 ± 18	75 ± 35[f]	418 ± 50[f]	71 ± 35[e]	89 ± 6	53 ± 2[f]	54 ± 9[f]	58 ± 9[e]	155 ± 7
mg/kg body wt				EXP. 2[c]					
0	97 ± 20	600 ± 92	145 ± 64	67 ± 21	95 ± 3	91 ± 4	81 ± 3	87 ± 5	57 ± 3
5	73 ± 11[d]	54 ± 18[f]	98 ± 38	49 ± 17	94 ± 5	74 ± 6[f]	66 ± 4[f]	70 ± 4[f]	44 ± 5[f]
15	60 ± 10[e]	17 ± 10[f]	64 ± 15[e]	26 ± 8[f]	96 ± 2	68 ± 1[f]	57 ± 3[f]	56 ± 8[f]	40 ± 6[f]

[a] Mean ± SD of 5 animals per group. Thymus and spleen cells were isolated from the whole organ, PLN cells from the pooled axillary and inguinal lymph nodes, BM cells from 1 and 2 femurs, respectively, in exp. 1 and 2.

[b] 9-Week-old male rats fed 0, 50, or 150 mg DOTC/kg diet from weaning age.

[c] 25–27-day-old rats given by gavage 3 times a week 0, 5, or 15 mg DOTC/kg body wt from day 2 after birth.

[d] $p < 0.05$.

[e] $p < 0.01$.

[f] $p < 0.001$.

Table 2 *Effect of organotin treatment of rats on the graft-versus-host reactivity of thymus, spleen and PLN cells*

Organotin dosage (mg/kg diet)	Cell for cell basis[a]			Whole organ basis[b]		
	thymus	spleen	PLN	thymus	spleen	PLN
	Exp. 1: Male rats exposed to DBTC for 31 days from weaning					
0	9.7 ± 2.5	26.5 ± 5.2	17.7 ± 6.0	100	100	100
50	8.8 ± 1.3	19.2 ± 5.7[c]	13.6 ± 5.4	64	57	47
150	9.0 ± 3.4[c]	18.6 ± 3.1[c]	13.3 ± 5.4[d]	24	40	32
	Exp. 2: Six-week-old female rats exposed pre- and postnatally to DOTC					
0	9.1 ± 1.4	31.2 ± 6.8		100	100	
50	14.0 ± 4.3[d]	23.2 ± 5.9[d]		49	53	
150	13.3 ± 2.9[d]	19.2 ± 5.2[d]		9	32	
	Exp. 3: Male adrenalectomized rats exposed to DBTC for 3 weeks following weaning					
0	16.0 ± 3.8			100		
150	20.1 ± 3.5			19		

[a] Mean values ± SD of PLN weights from groups of ten (WAG × B) F_1 recipients injected with 10×10^6, 3×10^6, or 1.5×10^6 viable nucleated cells from thymus, spleen, or PLNs, respectively, into the hind feet. In exp. 3, 18×10^6 thymocytes were transferred into the F_1 recipients.

[b] GVH reactivity per organ expressed as percentage of controls.

[c] $p < 0.01$.

[d] $p < 0.05$.

of spleen cells was significantly reduced both at the 50 and 150 ppm feeding level. The GVH activity of PLN cells was slightly but significantly reduced only at the highest dose level, whereas the GVH activity of thymus cells was not affected. Since DBTC treatment induced a marked lymphocyte depletion, the total GVH reactivity recoverable from the spleen and PLNs, as well as from the thymus, was significantly decreased at both feeding levels. After pre- and postnatal exposure to DOTC (Table 2, experiment 2) the effect on GVH activity was more pronounced. The total GVH activity of thymus was only 9 and 49% of the activity in control animals at the highest and intermediate dose level, respectively. However, on a cell for cell basis, the GVH activity of thymocytes was increased. Also, in adrenalectomized rats on a 150 mg DBTC/kg diet for 3 weeks, the GVH activity of thymus cells was slightly increased (Table 2, experiment 3), but on a whole organ basis there was a strong decrease.

Mitogenic Response of Lymphocytes from Di-*n*-Octyltindichloride-treated Rats

Littermate rats received 0, 5, or 15 mg DOTC/kg body weight by gavage for 4 weeks after birth. The mitogenic response of thymocytes to PHA and Con A was dose-relatedly decreased and approached the zero level (Fig. 1). A similar effect was seen in the response of spleen cells to PHA. A marked reduction was also found in spleen cells stimulated with Con A, but due to the large variation in values from control spleens, the difference was not statistically significant. Stimulation with LPS was the same in all groups. Thymidine incorporation of unstimulated cultures was not affected.

Survival of Rat and Human Thymocytes Exposed to Di-*n*-Butyltindichloride or Di-*n*-Octyltindichloride In Vitro

When rat thymocytes were cultured in the presence of graded amounts of DBTC or DOTC up to levels of 50 μg/ml medium, the survival of the cells was decreased in a dose-related and time-dependent fashion. The results of a typical experiment are given in Figure 2. Thymocyte

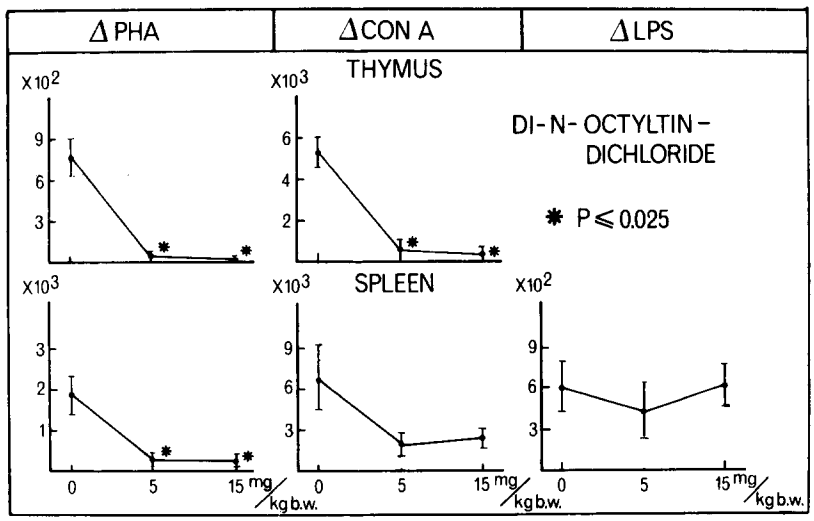
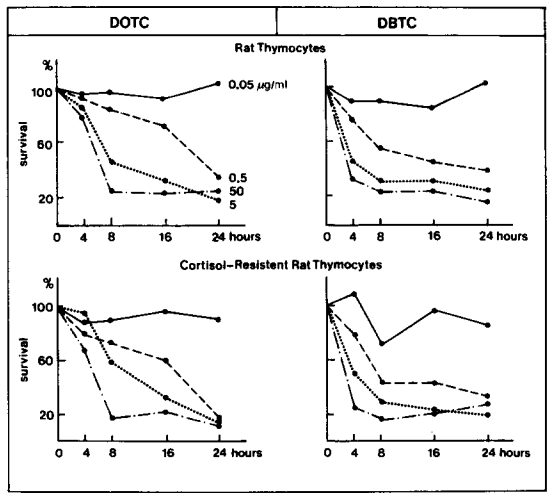


Figure 1 *Mitogenic response of thymus and spleen cells from littermate rats treated by gavage with 0, 5, or 15 mg DOTC, respectively, for 4 weeks after birth. Results are expressed as the difference of ^{3}H-TdR incorporation between stimulated and unstimulated cultures in cpm per million cells. (Mean ± 4–6 animals.)*

survival progressively decreased until, after 24 hr of incubation with 50 μg of DBTC or DOTC per ml, it was about 20% of the control value. At a concentration of 0.5 μg/ml thymocyte survival was still greatly decreased. At similar dose levels, a decreased thymocyte survival was seen with cortisol-resistant thymocytes (Fig. 2).

Human thymocytes incubated with DBTC and DOTC in the same manner as rat thymocytes at concentrations of 4×10^6 cells/ml aggregated in a dose-related fashion (Fig. 3). After 24 hr of incubation with 0.5 μg DBTC or DOTC/ml, about 50% of the thymocytes were aggregated.

Figure 2 *Survival of rat thymocytes and cortisol-resistant rat thymocytes upon exposure to various concentrations of DOTC and DBTC during different incubation periods. Results are expressed as percentage of the survival of the control cultures.*



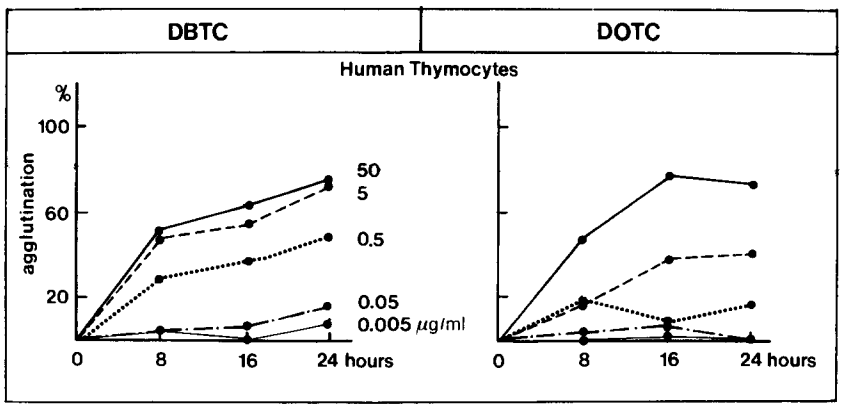


Figure 3 *Agglutination of human thymocytes upon exposure to various concentrations of DBTC or DOTC. Agglutination was calculated from the decline of cells counted electronically in the organotin-exposed cultures.*

Cell agglutination did not occur when the thymocytes were incubated with DOTC or DBTC in a shaking water bath at concentrations of 3×10^5 thymocytes/ml. In contrast to rat thymocytes, the survival of human thymocytes was not distinctly reduced by organotin treatment (see Figure 8). Cell survival was approximately 80% in cultures that contained 62.5 µg DOTC/ml or 0.5 µg DBTC/ml showing a marked difference in the lymphocytotoxic action of DOTC and DBTC for human thymocytes.

Blast Transformation of Rat and Human Thymocytes Exposed to Di-*n*-Butyltindichloride and Di-*n*-Octyltindichloride In Vitro

In rat thymocyte cultures containing various concentrations of DBTC or DOTC the incorporation of ^{3}H-thymidine was dose-relatedly decreased (Fig. 4). At concentrations of 0.1 µg DBTC and 0.5 µg DOTC per ml medium, thymidine incorporation was annihilated. At 5-fold lower concentrations a marked reduction was still seen, but after reducing the concentration another 5-fold, thymidine incorporation approached control values. The relative inhibition of thymidine incorporation by DBTC and DOTC was essentially the same in PHA, Con A, and nonstimulated cultures.

The effects of DBTC and DOTC on transformation of lymphocytes from spleen and thymus were similar (Fig. 4). The PHA and Con A, as well as the LPS response of rat splenocytes was equally affected by organotin exposure.

The inhibition of DNA synthesis was not dependent on the time of drug addition. If DBTC and DOTC were added over a 4-hr period before or 24 hr after the initiation of the mitogenic transformation, the same inhibition pattern of the ^{3}H-TdR incorporation was found (Fig. 5). However, total inhibition occurred at exposure levels 5-fold higher than those compared with results shown in Figure 4.

A dose-related inhibition of DNA synthesis also occurred in cortisol-resistant thymocytes cultured with increasing amounts of DBTC or DOTC (Fig. 6). However, at the same exposure levels, thymidine incorporation of cortisol-resistant thymocytes was less reduced. In comparison with normal thymocytes (Fig. 4), about the same degree of inhibition was found in cortisol-resistant thymocytes exposed to about 5-fold higher organotin levels.

DNA synthesis of human thymocytes exposed to DBTC or DOTC was also decreased in a dose-related fashion (Fig. 7). Thymidine incorporation was nearly zero at a concentration of 0.1 µg DBTC/ml, the same dose that annihilated the incorporation of rat thymocytes (Fig. 3). With DOTC, DNA synthesis of human thymocytes was completely inhibited at a level of 2.5 µg/ml.

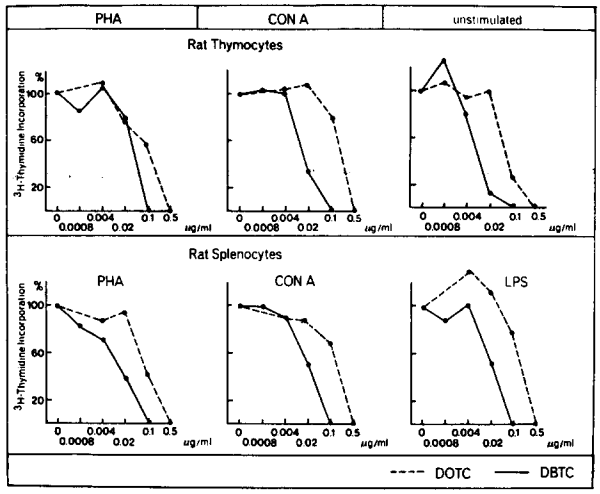


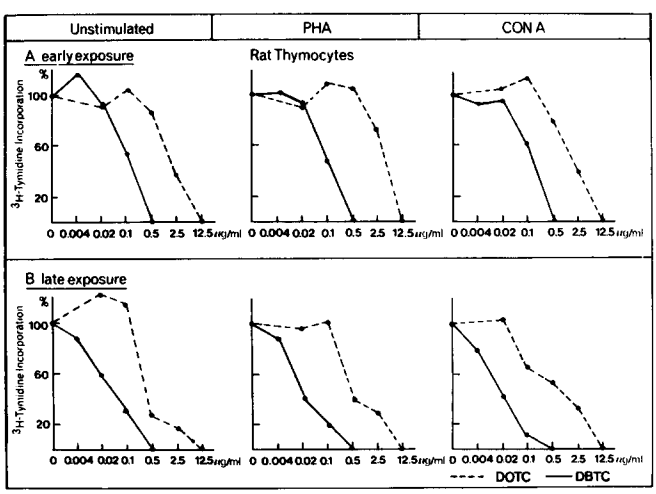
Figure 4 *In vitro effect of various concentrations of DBTC and DOTC upon the ^{3}H-TdR incorporation of rat thymus and spleen cells in PHA-, Con A-, and LPS-stimulated and unstimulated cultures. Results are expressed as percentages of the ^{3}H-TdR incorporation of the control cultures.*

A partial inhibition of the thymidine incorporation was already found at dose levels of 0.02 μg DBTC and 0.1 μg DOTC/ml.

E-rosette Formation of Human Thymocytes Exposed to Di-*n*-Butyltindichloride and Di-*n*-Octyltindichloride In Vitro

Upon incubation with DBTC or DOTC the ability of human thymocytes to form E-rosettes was impaired in a dose-related and time-dependent manner (Fig. 8). Di-*n*-butyltindichloride impaired the rosette formation after a shorter incubation time than did DOTC. Neither DOTC nor

Figure 5 *Time-dependency of drug addition on the ^{3}H-thymidine incorporation in PHA- and Con A-stimulated and unstimulated cultures of rat thymocytes. (A) Early exposure: the test compounds were only present for 4 hr preincubation. (B) Late exposure: DBTC and DOTC were added 24 hr after the initiation of the mitogen response.*



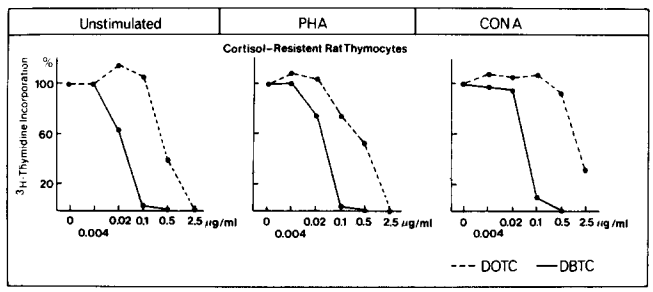


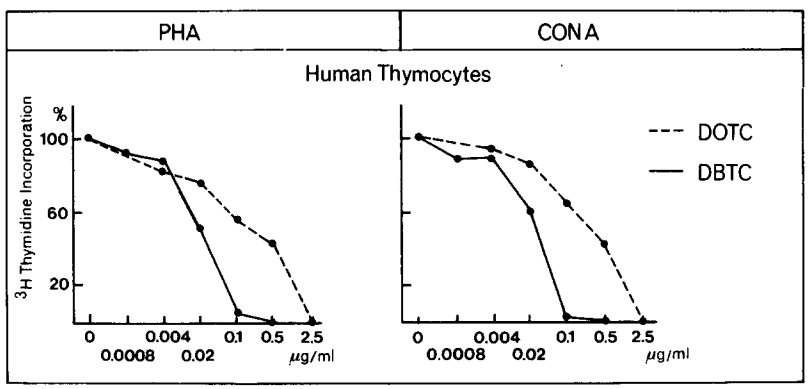
Figure 6 *In vitro effect of various concentrations of DBTC and DOTC upon the ^{3}H-TdR incorporation of cortisol-resistant rat thymocytes in PHA- and Con A-stimulated and unstimulated cultures. Results are expressed as percentage of the ^{3}H-TdR incorporation of the control cultures.*

DBTC diminished the rosette-formation ability after 10 min of incubation. After 4 hr the number of rosettes was markedly decreased by DBTC concentrations of 0.5 and 2.5 µg/ml, but was not distinctly affected by DOTC concentrations up to 62.5 µg/ml. After that, the number of rosette-forming thymocytes progressively decreased in the presence of both organotin compounds. Culturing of thymocytes in the presence of the drug for 22 hr reduced the number of rosettes at concentrations as low as 0.02 µg DBTC or 0.5 µg DOTC/ml. Rosette formation was almost completely inhibited at concentrations of 0.5 µg DBTC or 12.5 µg DOTC/ml; these concentrations did not diminish the viability of the cells.

DISCUSSION

Di-*n*-butyltin and di-*n*-octyltin compounds exhibit interesting antilymphocytic properties, since they induce atrophy of lymphoid organs, without signs of myelotoxicity and without compromising other organ systems (Seinen and Willems, 1976; Seinen et al., 1977a; Seinen and Penninks, 1979). Thymus was the most severely affected organ. In rats administered oral doses of 5 and 15 mg DOTC/kg body weight, three times a week from day 2 after birth until day 25, the thymus contained 9 and 3% of the number of lymphocytes of control animals, respectively. The number and viability of cells from spleen and lymph nodes was also dose-relatedly decreased by this treatment. Although the number of cells isolated from bone marrow of rats of the 15 mg

Figure 7 *In vitro effect of various concentrations of DBTC and DOTC upon the ^{3}H-TdR incorporation of human thymocytes in response to PHA and Con A. Results are expressed as percentages of the ^{3}H-TdR incorporation of the control cultures.*



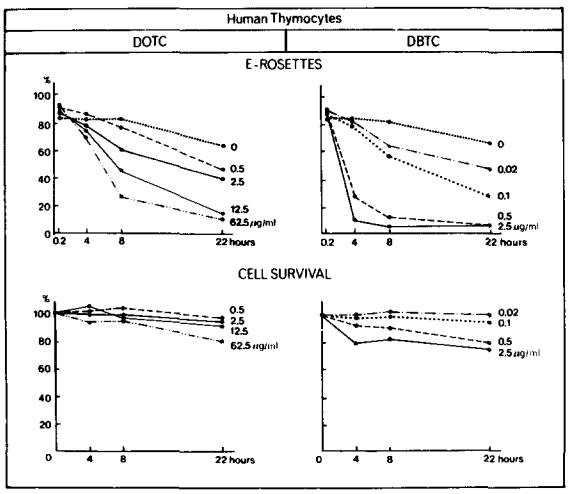


Figure 8 *In vitro effect of various concentrations of DBTC and DOTC upon the E-rosette formation and survival of human thymocytes.*

DOTC/kg group was decreased, the viability of these cells was not reduced. When DOTC was fed to rats at dietary levels of 50 or 150 mg/kg, concentrations that did not reduce body weight, the total number and viability of bone marrow cells was comparable with the control values, whereas the number and viability of cells from the other lymphoid organs were significantly decreased. Therefore, the reduction of bone marrow cells found after postnatal treatment with DOTC (Table 1) is considered to be a reflection of decreased body weights and is not caused by a cytotoxic action of DOTC. Also with the Till and McCulloch assay (1961) no effects of BM cells were observed. The number of 10-day spleen colonies produced in lethally irradiated recipient mice injected with BM cells of donor mice treated with 0,2 or 10 mg DBTC/kg intravenously (iv) were essentially the same (Seinen and Penninks, 1979). These results indicate that DBTC and DOTC in vivo produce selective cytotoxic effects in thymus and peripheral lymphoid organs.

In vitro, DOTC and DBTC are extremely cytotoxic for lymphocytes. Concentrations of 0.5 µg/ml medium effectively decreased the survival of rat thymic lymphocytes. The survival of thymocytes from normal and cortisol-treated rats was equally decreased. In a previous study it was shown that the survival of rat BM cells was not reduced by exposure to 100-fold higher concentrations of DBTC (Seinen et al., 1977b).

Suppression of cell-mediated immunity by DBTC and DOTC, as previously reported (Seinen et al., 1977b), is further supported by the reduction of GVH reactivity recoverable from thymus, spleen, and PLNs. On a cell for cell basis the GVH reactivity of spleen and PLN cells was decreased by DBTC or DOTC, whereas the GVH indices of thymus were higher. As the lymphocytes responsible for the initiation of the GVH reactions have been shown to be relatively resistant to corticosteroids (Blomgren and Andersson, 1969; Tigelaar and Asofsky, 1973), the higher GVH indices of thymocytes could be explained by a stress-induced release of glucocorticosteroids. However in adrenalectomized and normal organotin-treated rats a comparable increase of GVH activity was found on a cell for cell basis. Also, in a previous study (Seinen and Willems, 1976), it was found that the thymolytic effect of DOTC was not affected by adrenalectomy. Therefore it is concluded that the increase in GVH activity of thymocytes found in organotin-treated animals is not mediated by an increased corticosteroid production. Yet, DOTC treatment may cause some differences in the loss of thymocytes reactive and nonreactive in the GVH assay. However, this difference is much less impressive than in case of corticosteroids. Blomgren and Andersson (1971) and Cohen and Claman (1971) suggested that the vast majority of the thymo-

cytes competent to initiate GVH reactions were corticosteroid resistant. Tigelaar and Asofsky (1973) found a 50% loss of total GVH reactivity recoverable from the thymus in mice with 125 mg cortisone acetate/kg body weight. In our study the loss of the total GVH reactivity recoverable from rat thymus was 80–90% after dietary treatment with 150 mg of the organotin compound/kg diet. The total GVH activity recoverable from the spleen and PLN was 60–70% decreased. Thus it appears that both GVH reactive and GVH nonreactive cells are eliminated from the lymphoid tissues by DBTC and DOTC treatment.

The presence of T-cell suppression is also indicated by the inhibition of blast transformation of thymus and spleen cells from DOTC-treated rats upon stimulation with the mitogens PHA and Con A. In contrast the ^{3}H-TdR incorporation of spleen cells upon *E. coli* LPS was not impaired by DOTC treatment. The antibody response against this B-cell antigen was not reduced by organotin treatment, whereas the humoral response against SRBC was inhibited, as indicated by a reduction of plaque-forming spleen cells and decreased hemagglutination titers (Seinen et al., 1977b). Therefore it is concluded that only T lymphocytes are impaired by DBTC and DOTC; this is in agreement with the lymphocyte depletion observed selectively in thymus and thymus-dependent areas of the peripheral lymphoid organs of rats exposed to these drugs (Seinen and Willems, 1976; Seinen et al., 1977a). In vitro, concentrations of 0.02 μg DBTC and 0.1 μg DOTC/ml medium reduced the ^{3}H-TdR incorporation of rat lymphocytes. Blast transformation of rat lymphocytes was completely inhibited by 0.1 μg DBTC and 0.5 μg DOTC/ml medium.

Since thymidine incorporation was proportionally inhibited in PHA, Con A, LPS, and unstimulated cultures, this effect is not mediated by a chemical interaction of the drugs with mitogens. However, it does indicate a general antiproliferative effect of the organotin compounds, while showing no evidence of the differential effects upon T cells found after in vivo treatment.

Inhibition of blast transformation was not dependent upon the time of drug addition. If the drugs were only present for 4 hr before or were added 24 hr after initiation of the mitogenic transformation, they were slightly less effective, probably because of a lower intracellular concentration of the drugs.

Between the cortisol-resistant and normal thymocytes slight differences in DBTC and DOTC sensitivity were observed. Inhibition of the mitogenic response of cortisol-resistant thymocytes occurred at 5-fold higher drug concentrations than with normal thymocytes. This may possibly explain the increase of GVH reactivity of thymocytes from organotin-treated animals.

Between rat and human thymocytes a marked similarity was found. Although the viability of human lymphocytes was less reduced than rat lymphocytes, the human cells were definitely damaged by the tin compounds. They lost their ability to form E-rosettes with SRBC and showed a tendency to aggregate upon exposure to DOTC and DBTC. Inhibition of E-rosette formation was a very sensitive parameter of drug action. Concentrations as low as 0.02 μg DBTC and 0.5 μg DOTC/ml already decreased the formation of E-rosettes. Other immunosuppressive drugs, such as azathioprine and amethophterin diminished the E-rosette-forming ability of human lymphocytes at 100- and 1000-fold higher concentrations, respectively (Bach, 1969). In comparison with other immunosuppressive drugs, the blast transformation also was inhibited at very low concentrations of DBTC and DOTC (Bach, 1975). ^{3}H-thymidine incorporation of human as well as rat thymocytes was inhibited at levels of 0.02 μg DBTC and 0.1 μg DOTC/ml medium. These results indicate a comparable effect of organotin compounds upon human and rat lymphocytes.

From a toxicological point of view DBTC and DOTC are hazardous compounds. In our opinion, the use of organotins (e.g., in food packing plastics) (Piver, 1973) has to be reevaluated, since toxicological evaluation of these compounds was done at a time when the immunosuppressive properties were not known. Especially since the effects on the lymphoid system are the most sensitive parameters of DBTC and DOTC toxicity.

From a pharmacological point of view, these compounds represent a potential tool for controlling various pathological events in which undesired T-lymphocyte functions are involved, since DBTC and DOTC induce immunosuppression selectivity in those test systems where

T lymphocytes are predominantly involved. Moreover, in contrast to many other immunosuppressive drugs, DBTC and DOTC do not exert myelotoxicity or a generalized cytotoxicity.

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