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The organotin-induced thymus atrophy, characterized by depletion of $CD4^+CD8^+$ thymocytes, is preceded by a reduction of the immature $CD4^-CD8^+TcR\alpha\beta^{-/low}CD2^{high}$ thymoblast subset

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SUMMARY

Thymic changes in the rat induced by the thymus atrophy-inducing organotin compound di-*n*-butyltin dichloride (DBTC) were examined using FACS analyses. The number of $CD4^+CD8^+$ thymocytes was reduced by DBTC treatment from Day 2 onwards and reached minimum level on Days 4 and 5 after dosing. On these days the $CD4^-CD8^-$ and both the $CD4^-CD8^+$ and $CD4^+CD8^-$ subsets were not affected. On Day 2 we observed a reduced proportion of transferrin receptor (CD71)-positive $CD4^-OX44^-$ cells, representing the cycling immature $CD4^-CD8^+$ cells, and of $CD71^+OX44^-$ cells, representing the cycling $CD4^+CD8^+$ cells, but not of $CD71^+CD4^-CD8^-$ cells. When compared to controls, the FSC^{high} cell population of DBTC-treated rats contained less $CD4^-OX44^-$ and $OX44^-$ cells, which were further characterized as $CD2^{high}$ and T-cell receptor $(TcR)\alpha\beta^{-low}$. Moreover, fewer $TcR\alpha\beta^{high}$ cells were detected in the $OX44^-$ thymoblast subset of DBTC-treated rats. The number of $CD4^-CD8^-$ thymoblasts appeared marginally decreased while the numbers of $CD4^+OX44^+$ cells, representing mature $CD4^+$ cells, were not affected. These data indicate that DBTC causes a preferential initial depletion of immature $CD4^-CD8^+CD2^{high}$ $TcR\alpha\beta^{-low}$ thymoblasts. This initial event may result in a decreased formation of $CD4^+CD8^+$ thymoblasts and of small $CD4^+CD8^+$ thymocytes. These characteristics of the initially depleted subset indicate a specific anti-proliferative effect of DBTC and may give clues for the mechanism involved in the induction of thymus atrophy.

INTRODUCTION

Di-*n*-butyltin dichloride (DBTC) is a dialkylated organotin compound that is well-known for its toxic effect on the immune system. Dialkyltin derivatives are used as PVC-stablizers and industrial catalysts,[1] and hence have a wide application. The most prominent effect of DBTC and some other dialkyltins is a dose-dependent and reversible reduction of thymus weight and has been observed in rats after a single[2] as well as prolonged[3] oral exposure. After prolonged exposure, these dialkyltins also appeared to induce a depletion of peripheral T lymphocytes and to suppress various T-cell-dependent immune responses.[4,5] Despite much effort, the mechanism by which these chemicals induce thymus atrophy still remains unclear. Histological examination of the thymus showed that dialkyltins caused atrophy of the thymic cortex in the absence of cell destruction.[3] This observation together with the finding that the compounds also induce thymus atrophy in adrenalectomized rats[3] indicate that stress is not involved. More detailed data on the mechanism of the DBTC action were obtained by daily analysis of thymocyte size distribution and the capacity of thymocytes to synthesize DNA after a single oral treatment.[2] It was found that both the number of blastoid thymocytes and total thymocyte proliferation were already maximally reduced by 24–48 hr after treatment, but normal again on Day 4. Moreover, the number of transferrin receptor (CD71)-positive thymocytes was found to decrease with the same kinetics as the thymoblasts.[6] The initial decrease of proliferating thymocytes was followed by a marked reduction of small thymocyte numbers and thymus weight that reached minima on Days 4 and 5 and had recovered on Day 9.[2] Based on these data DBTC-induced thymus atrophy has been attributed to an anti-proliferative effect.

In recent years knowledge of thymocyte differentiation in the rat[7–11] has increased rapidly. With regard to the CD4 and CD8 markers, differentiation proceeds from immature $CD4^-CD8^-$ thymocytes through an intermediate stage of immature $CD4^-CD8^+$ cells to $CD4^+CD8^+$ cells which finally differentiate to CD4 or CD8 single positive mature thymocytes. A more detailed hypothetical model for thymocyte differentiation, based on ontogeny studies and involving many lymphoid cell markers, has been proposed by Kampinga and Aspinall.[10,11] This knowledge facilitated further characterization of thymic changes induced by DBTC.

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Previous immunohistological examinations showed a successive, time-dependent decrease of $CD2^+$, $CD8^+$, $CD4^+$, and $CD5^+$ cells and an increase of acid phosphatase-negative $OX44^+$ cells in the cortex.[12] In the thymus cortex the OX44 marker is present on all leucocytes, immature $CD4^-CD8^+$ and $CD4^+CD8^+$ thymocytes excepted.[7,8] Therefore, the cortical $OX44^+$ cells are likely to represent $CD4^-CD8^-$ thymocytes. This indicated that DBTC interfered with the differentiation of immature thymocytes beyond the earliest $CD4^-CD8^-$ stage. However, these cortical $OX44^+$ cells could also represent mature medullary cells in an atrophied cortex.

In this study, FACS analyses were performed to quantify sequential changes in CD4/CD8 subsets in the DBTC-induced thymus atrophy. Moreover, the thymoblasts that were initially depleted on Day 2 were characterized in more detail.

MATERIALS AND METHODS

Animals and treatment

Male Wistar-derived rats were obtained from Harlan/CPB (CPB-Wu), Austerlitz, The Netherlands, or from the breeding facilities of the Central Animal Laboratory (U-Wu), University of Utrecht, The Netherlands. The rats, aged 5–6 weeks and weighing 94 ± 4 g at the start of the experiments, were housed under standard conditions (temperature $23 \pm 2^\circ$, 50–60% relative humidity, 12 hr light/dark cycle). DBTC was kindly provided by Dr H. A. Meinema (Institute for Applied Chemistry, TNO, Zeist, The Netherlands), and the purity was over 99%. DBTC was administered by gavage as a 5% ethanol solution in corn oil at a dose of 0 or 15 mg/kg body weight and in a volume of 2·5 ml/kg. At Days 1, 2, 3, 4, 5, 7 and 9 after gavage, control and DBTC-exposed rats were exsanguinated by decapitation and thymi were removed. Thymus glands were carefully trimmed free from adjacent lymph nodes before they were used to prepare thymocyte suspensions.

Monoclonal antibodies

The ER2 (anti-CD4, IgG1)-hybridoma[9] was kindly provided by Dr J. Rozing (TNO, Leiden, The Netherlands). The cell lines MRC OX8 (anti-CD8, IgG1)[13], MRC OX34 (anti-CD2, IgG2a)[14], MRC OX26 (anti-CD71, IgG2a)[15] and MRC OX44 (anti-CD53, IgG1)[7,8,16] were obtained from the European Collection of Animal Cell Cultures, (ECACC), Salisbury, U.K. The R-73 [anti-T-cell receptor (TcR), pan $\alpha\beta$, IgG1] cell line[17] was kindly provided by Dr T. Hünig (Würzburg, Germany). MRC OX8, MRC OX44, and ER2 were biotinylated by Mr R. van Herwijnen (European Veterinary Laboratory, Amsterdam, The Netherlands). High-performance liquid chromatography (HPLC)-purified monoclonal antibodies (mAb) ER-2 and OX8 were coupled to fluorescein isothiocyanate (Sigma Chemical Co., St Louis, MO), according to standard procedures.[18]

Preparation of thymocyte suspensions and FACS stainings

Thymocytes were isolated as previously described.[2] Briefly, thymocytes were pressed through a nylon sieve, washed three times in ice-cold Dulbecco's phosphate-buffered saline (PBS) supplemented with 2 mM D-glucose (pH 7·4) and resuspended in ice-cold PBS containing 10% heat-inactivated foetal calf serum and 0·1% NaN_3. Numbers of thymoblasts, i.e. cells with a diameter over 8·2 μm, were determined on a Coulter Counter ZM (Coulter Electronics, Luton, Beds, U.K.) set for the appropriate threshhold value. Finally, total cell concentration was adjusted to 4×10^7 cells/ml.

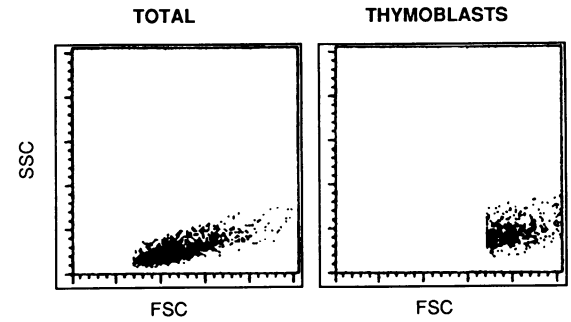

Figure 1. Analysis of total and FSC^{high} cells by setting gates in FSC (forward scatter) versus SSC (side scatter) dot plots. The gate for FSC^{high} cells was set in such a manner that the average percentages of these cells and of cells with a diameter over 8·2 μm was the same. The proportion of cells with a diameter over 8·2 μm was determined with the Coulter Counter (see Materials and Methods).

Cells were stained for 30 min at room temperature with combinations of biotinylated and fluorescein isothiocyanate (FITC)-conjugated mAb. Streptavidin-phycoerythrin (PE) (Southern Biotechnology Association, Inc., SBA, Birmingham, AL) was used to visualize biotin. In case of stainings in combination with OX34 and OX26, FITC-conjugated goat anti-mouse IgG2a (SBA) was used as a second step. Total thymocytes were subdivided into a low ($CD2^{low}$, $\pm 75\%$ of total) and a high ($CD2^{high}$, $\pm 25\%$ of total) positive subset by staining with OX34. Double stainings with unconjugated R73 were performed according to a five-step staining procedure. Cells were successively incubated with R73 supernatant, FITC-conjugated goat anti-mouse IgG1 (SBA), 20% normal heat-inactivated mouse serum (20 min), biotinylated mAb and streptavidin-PE. The staining pattern of R73 was as originally described.[17] All SBA conjugates were used in the presence of 5% normal heat-inactivated rat serum. Between all staining steps cells were washed with PBS, 1% bovine serum albumin (BSA), 0·1% NaN_3.

Per sample, 3×10^3 cells were acquired on a FACScan flow cytometer (Becton Dickinson, Erembodegem, Belgium). To determine the phenotype of thymoblasts, cells were analysed with a gate set for FSC^{high} cells (Fig. 1). The FSC^{high} cells thus analysed made up the same proportion of the total thymocyte population as the large cells with a diameter over 8·2 μm.

RESULTS

Sequential changes in CD4/CD8 subsets and phenotypes of total thymocytes on Day 2

The number of $CD4^+CD8^+$ thymocytes decreased from Day 2 and reached minimum level on Day 5 after exposure to DBTC (Fig. 2). On that day, thymi of DBTC-treated rats contained 1×10^8 $CD4^+CD8^+$ cells representing about 60% of all thymocytes while control thymi contained 6×10^8 $CD4^+CD8^+$ cells, representing about 80% of all thymocytes. Percentages of $CD4^-CD8^-$ and of $CD4^+CD8^-$ and $CD4^-CD8^+$ were increased to about 10%, 16% and 13%, respectively (Fig. 3). Absolute numbers of these phenotypes, however, were not significantly changed on Day 5 (Table 1). On Day 7, the number

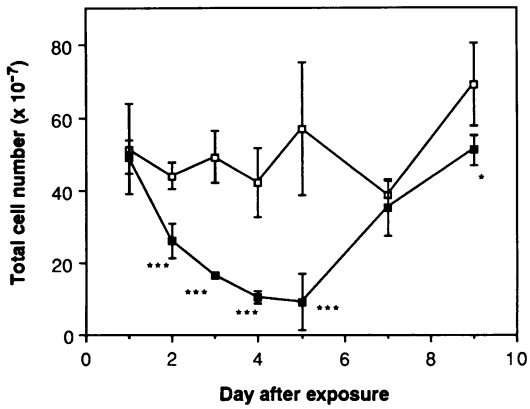

Figure 2. Effect of a single oral dose of 15 mg DBTC/kg body weight on the number (mean ±SD) of $CD4^+CD8^+$ thymocytes on various days after exposure. Open symbols represent controls and closed symbols represent DBTC-treated rats. $*P<0.05$, $***P<0.001$ in Student's *t*-test ($n=6$).

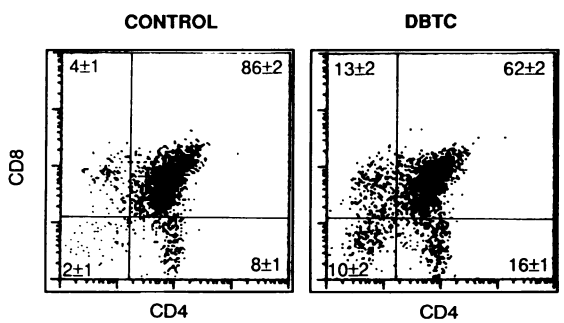

Figure 3. Two-colour (CD4/CD8) FACScan analyses of thymocytes obtained from control or DBTC-treated rats on Day 5 after dosing. FACS diagrams shown are of representative animals, while percentages are given as the mean ±SD of four animals.

Table 1. The number of total CD4/CD8 subsets per whole thymus of control and DBTC-treated rats

	Number of thymocytes ±SD ($\times 10^{-7}$)			
	Day 2		Day 5	
Phenotype	Control	DBTC†	Control	DBTC
$CD4^-CD8^-$	1·1±0·1	0·7±0·2**	1·4±0·7	1·6±1·1
$CD4^-CD8^+$	4·2±0·7	2·7±1·1*	2·8±1·5	2·4±1·8
$CD4^+CD8^-$	4·1±0·8	3·0±1·1	5·0±1·3	2·9±2·0
$CD4^+CD8^+$	44·0±3·7	26·1±4·7***	57·0±18·2	9·0±7·9**

† Rats were exposed to a single oral dose of 15 mg DBTC/kg body weight.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$ in Student's *t*-test.

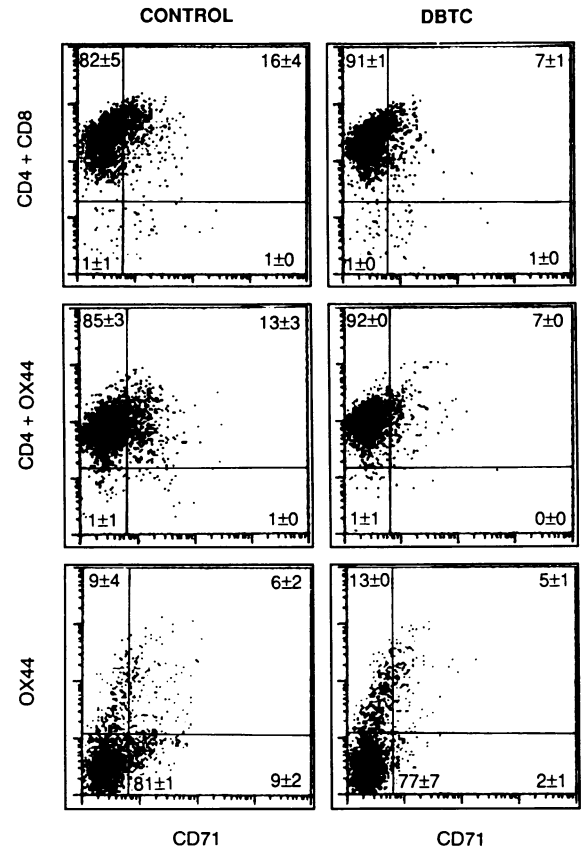

Figure 4. Staining for CD71 combined with CD4 plus CD8, CD4 plus OX44 or OX44 alone of total thymocyte populations obtained from control and DBTC-treated rats 2 days after dosing. FACS diagrams shown are of representative animals, while percentages are given as the mean ±SD of three animals.

of $CD4^+CD8^+$ cells (Fig. 2) and of the other CD4/CD8 subsets (not shown) reached control values again.

On Day 2, the proportions of the $CD4^+CD8^+$(±83%), $CD4^-CD8^-$(±2%), and the single positive $CD4^+CD8^-$ (±12%) and $CD4^-CD8^+$(±4%) subsets in total thymocyte suspensions were not changed by DBTC. However, besides the clear decrease in the total number of $CD4^+CD8^+$ thymocytes on Day 2, the numbers of $CD4^-CD8^-$ and of $CD4^-CD8^+$ cells were slightly reduced (Table 1). The relative numbers of the $CD4^-OX44^-$ thymocytes (±2% in controls and in DBTC-treated rats) and of the other thymocyte subsets identified by CD4/OX44 staining were not influenced as well. Because OX44 is expressed by all thymocytes except the immature $CD4^-CD8^+$ cells and virtually all $CD4^+CD8^+$ cells,[12,15] $CD4^-OX44^-$ thymocytes can be considered as immature $CD4^-CD8^+$ cells. Moreover, the observation that the $CD4^+OX44^-$ subset (±85% of total thymocytes) outnumbers the $CD4^-OX44^-$ subset (±2% of total thymocytes) by far indicates that quantification of all $OX44^-$ cells gives a good measure of the double-positive subset.

Phenotype of $CD71^+$ cells and FSC^{high} thymocytes on Day 2

Staining for the CD71 with mAb OX26, combined with CD4/CD8, CD4/OX44 or OX44 alone, showed a decrease of the

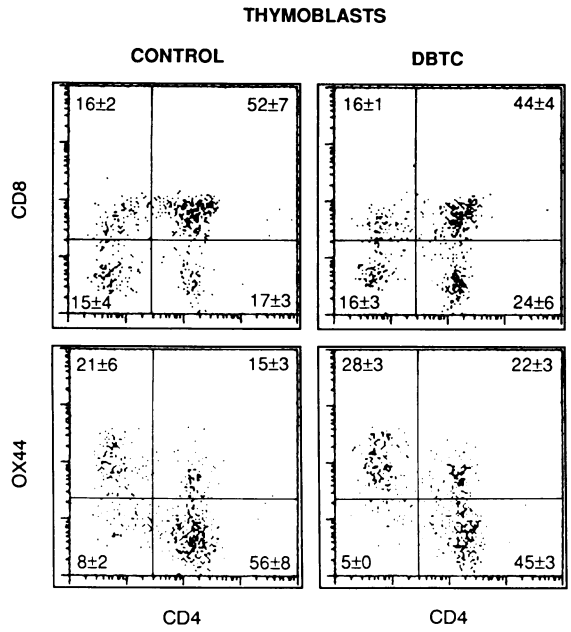

Figure 5. Analyses of FSC^{high} thymocytes after two-colour CD4/CD8 and CD4/OX44 staining of total thymocyte suspensions obtained from control or DBTC-treated rats on Day 2 after dosing. FACS diagrams shown are of representative animals, while percentages are given as the mean ±SD of five animals.

proportions of $CD71^+CD4^+OX44^+$, $CD71^+CD4^-OX44^-$ and $CD71^+OX44^-$ phenotypes and of $CD71^+$ cells that are also positive for CD4 and/or CD8. The percentage $CD71^+CD4^-CD8^-$ thymocytes was not decreased (Fig. 4).

In addition to the analyses for CD71-positivity, the phenotype of FSC^{high} cells, considered to represent thymoblasts, were analysed for their antigen expression. Comparison of the total control thymocyte population with the thymoblast population showed a decrease of the $CD4^+CD8^+$ and an enrichment of both CD4 and CD8 single positives, and of the $CD4^-CD8^-$ subset (Fig. 3 and 5, left panels). The proportion of $CD4^+OX44^-$ cells, representing $CD4^+CD8^+$ thymocytes, was decreased from 88±2% in the total thymocyte population to 56±8% in the thymoblast population, while the proportion of immature $CD4^-CD8^+$ cells was enhanced from 2±0% in total thymocyte population to 8±2% in thymoblast population.

Treatment with DBTC tended to increase the proportion of $CD4^+CD8^-$ blasts (Fig. 5, upper panels) which agrees with the DBTC-induced relative increase of $CD4^+OX44^+$ blasts (Fig. 5, lower panels). Absolute numbers of this latter subset remained unaffected, i.e. $3{\cdot}4 \pm 1{\cdot}9 \times 10^6$ in control thymus and $2{\cdot}3 \pm 0{\cdot}8 \times 10^6$ in DBTC-treated thymus. DBTC, however, clearly decreased the relative and absolute numbers of $CD4^-OX44^-$ and $CD4^+OX44^-$ blasts (Fig. 5, Table 2). These data indicate that DBTC treatment preferentially decreases proliferating cells of the immature $CD4^-CD8^+$ and $CD4^+CD8^+$ phenotype.

CD2 and TcRαβ expression on the thymoblasts

Additional CD2 staining showed that almost all immature $CD4^-CD8^+(CD4^-OX44^-)$ and the majority of the $CD4^+CD8^+(OX44^-)$ thymoblasts expressed high levels of CD2 and that these $CD2^{high}$ subpopulations were very markedly reduced after treatment with DBTC (Table 2). Staining for TcRαβ indicated that virtually all immature $CD4^-CD8^+(CD4^-OX44^-)$ and about two-thirds of the $CD4^+CD8^+(OX44^-)$ thymoblasts had the $TcR\alpha\beta^{-/low}$ phenotype whereas one-third of these $CD4^+CD8^+(OX44^-)$ cells carried high amounts of TcRαβ. All these three subsets were profoundly reduced by DBTC (Table 2). The majority $CD4^-CD8^-$ thymoblasts expressed also the $TcR\alpha\beta^{-/low}$ phenotype but the number of these cells were not affected. Although some decrease of total and $CD2^{low}CD4^-CD8^-$ subsets was observed these effects appeared to be very unclear and less consistent than in the case of the immature $CD4^-CD8^+(CD4^-OX44^-)$ phenotypes.

DISCUSSION

Previous studies showed that a single oral dose of DBTC induced a reduction of proliferating thymoblasts followed by a profound decrease of small cortical thymocytes and thymus weight.[2,6] The present study shows that the number of $CD4^+CD8^+$ thymocytes decreased with the same kinetics as the small-sized thymocytes. Both reached minima on Days 4 and 5 and attained control level again on Day 7 after dosing. Total numbers of other CD4/CD8-characterized subsets hardly changed with DBTC treatment. Therefore, it can be concluded that the DBTC-induced thymus atrophy results from a selective depletion of cortical, small-sized $CD4^+CD8^+$ thymoctyes.

As a consequence of the selective depletion of $CD4^+CD8^+$ cells the proportions of the $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ subsets were increased on Day 5. The acid phosphatase-negative $OX44^+$ cells, which have been found to be increased in the cortex after DBTC treatment,[12] are thus likely to be of the $CD4^-CD8^-$ subset. These FACS data confirm the findings obtained by immunohistology that DBTC interferes with early thymocyte differentiation and the data extend these findings by indicating that the transition from the $CD4^-CD8^-$ to the $CD4^+CD8^+$ stage is disturbed.

The depletion of thymoblasts on Days 1 and 2 is thought to be the initial event in the DBTC-induced thymus atrophy.[2,6] To determine the phenotype of these initially depleted thymoblasts, the phenotype of $CD71^+$ as well as of FSC^{high} thymocytes of DBTC-treated rats were compared with those of vehicle-treated rats for their expression of various antigens. Disappearance of $CD71^+$ cells was detected within the immature $CD4^-CD8^+(CD4^-OX44^-)$ and in the $CD4^+CD8^+(OX44^-)$ subsets but not in the $CD4^-CD8^-$ subset. Among the FSC^{high} cells a prominent depletion was observed of both $CD2^{high}$ and $TcR\alpha\beta^{-low}$ immature $CD4^-CD8^+(CD4^-OX44^-)$, and of both $CD2^{high}$ and $TcR\alpha\beta^{/low}CD4^+CD8^+(OX44^-)$ cells. In addition, the thymoblast population contained a reduced number of $CD4^+CD8^+(OX44^-)TcR\alpha\beta^{high}$ cells. According to the single lineage model of rodent thymocyte differentiation, in which $CD4^+CD8^+$ cells are considered to descend directly from the immature $CD4^-CD8^+$ subset,[8,10,11,19-21] DBTC probably preferentially depletes the thymoblast subset with the $CD4^-CD8^+OX44^-TcR\alpha\beta^{-/low}CD2^{high}$ phenotype.

Based on a calculated cycling time of proliferating thymocytes of 7–9 hr,[22] depletion of this small blast subset on Day 2 may well explain the profound reduction of small $CD4^+CD8^+$ thymocytes observed on Day 5. During the 3-day interval

Table 2. The number of thymoblast subsets per whole thymus of control and DBTC-treated rats on Day 2 after dosing

	Number of thymoblasts per thymus ±SD ($\times 10^{-6}$)					
	$CD4^-CD8^-$		$CD4^-OX44^-$ (immature $CD4^-CD8^+$)		$OX44^-$ ($CD4^+CD8^+$)	
	Control	DBTC†	Control	DBTC	Control	DBTC
Total	2·9±0·9	1·7±0·7*	1·6±0·5	0·5±0·2**	ND	ND
$CD2^{low}$	0·8±0·3	0·3±0·1*	0·0±0·0	0·0±0·0	0·6±0·1	1·0±0·5
$CD2^{high}$	2·0±0·5	1·5±0·7	1·7±1·1	0·4±0·1*	12·6±6·6	4·0±1·6*
$TcR\alpha\beta^{-/low}$	2·0±0·9	1·3±0·7	1·7±1·2	0·5±0·2*	10·4±3·3	2·1±1·2***
$TcR\alpha\beta^{high}$	0·2±0·1	0·1±0·0	0·7±0·4	0·5±0·2	6·7±2·2	2·9±1·7*

† Rats were exposed to a single oral dose of 15 mg DBTC/kg body weight.
* $P<0·05$, ** $P<0·01$, *** $P<0·001$ in Student's *t*-test.
ND, not determined.

1×10^6 immature $CD4^-CD8^+(CD4^-OX44^-)$ cells that are eliminated by DBTC would have given rise to $26–125\times10^7$ descendants, being of the same order of magnitude as the number of depleted small $CD4^+CD8^+$ cells.

The mechanism by which DBTC depletes immature thymoblasts is not known. *In vitro* studies showed a reduction of spontaneous and mitogen-induced thymocyte proliferation by DBTC, but also of mitogen-induced splenocyte proliferation.[4] The finding that the number of $CD4^+OX44^+$ thymoblasts, representing mature $CD4^+CD8^-$ cells, seemed unaffected indicates that instead of a mere anti-proliferative effect a more specific mode of action of DBTC is probably at work.

The high expression of CD2 on the preferentially depleted thymoblasts, in view of the earlier observed inhibitory effect of DBTC on human thymocyte/SRBC-rosette formation[4] may give a clue to the mode of action of the organotin compound. It suggests that DBTC can disturb the CD2/LFA3 interaction which is involved in the afore-mentioned rosette formation[23] and which may be of importance in interactions of immature human thymocytes with thymic epithelial cells.[24–27] However, since the role of CD2/LFA3 in rodents is even less clear than in man,[28,29] conclusions on a potential interference of DBTC with CD2/LFA-3 interactions in rodent thymi cannot yet be drawn.

Part of the initially depleted immature $CD4^-CD8^+$ thymoblasts expressed low levels of $TcR\alpha\beta$. However, cross-linking of these few receptors may very well prevent differentiation to the $CD4^+CD8^+$ phenotype as shown by Hünig.[30] Since DBTC has strong affinity for dithiol groups it may serve as a cross-linking compound.[31] With regard to this option it may be of interest that *in vivo* treatment of mice with antibodies against the CD3-$TcR\alpha\beta$ complex resulted in a preferential decrease of thymocytes that express low levels of CD3.[32] Moreover, the changes in the CD4/CD8 subsets observed in that study resemble those seen with DBTC.

Although the mechanism of DBTC toxicity is still unclear, it is obviously different from that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a more notorious environmental pollutant also capable of inducing thymus atrophy. The latter agent may cause atrophy by disturbing thymic epithelial cell function as well as by a direct effect on stem cells in the bone marrow (reviewed in ref. 33).

Further investigation of the mechanism of the DBTC-induced thymus atrophy is necessary to elucidate the role of CD2 and $TcR\alpha\beta$. Examination of the selective thymus effects of DBTC will not only add to a better understanding of the specific mode of action of organotins but also, like other agents such as TCDD and cyclosporin A, provide answers to fundamental questions with regard to thymocyte development.[33]

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