No Immunotoxic Effect on T Cells with Di (2-Ethylhexyl) Phthalate in Male C57BL/6 Mice

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Abstract

Objectives: To clarify whether di (2-ethylhexyl) phthalate (DEHP) has immunotoxic effects on both the expression of surface molecules (CD3, CD4, CD8 and CD28) on T cells of the thymus and spleen in male C57BL/6 mice.

Methods: Animals were orally administered a 0.1% or 0.2% DEHP-containing diet for 10 or 20 days. Dietary corn oil was used as the vehicle for DEHP in preparing the diet.

Results: Significant hepatic hypertrophy was clearly observed in the DEHP-exposed groups, while no atrophy was seen in the thymus or spleen in any treatment groups. In the thymus and spleen, no variation in the proportions of both T cells expressing CD3, CD4 and CD8 was shown with cytometry analysis. The surface expression of CD3, CD4, CD8 and CD28 on both T cells was also invariable in all analyzed stages of thymic differentiation and in the spleen. No effect of DEHP on mitogenesis was shown in the splenic T cells with an *in vitro* [³H]-thymidine-incorporation technique.

Conclusions: DEHP is probably not an immunosuppressor, particularly for T cells.

Key words: di (2-ethylhexyl) phthalate, immunotoxicity, T cells, thymic differentiation, CD antigens

Introduction

Di (2-ethylhexyl) phthalate (DEHP) is a dialkyl phthalate that is used as a plasticizer for polyvinyl chloride. Plasticized polyvinyl chloride is used in various materials such as a component of tubing, films, toys, and electric cables, indispensable for the development of modern industry, household products, etc. Currently, there is increased toxicological awareness for utilization of DEHP, since the chemical is known to be carcinogenic (1, 2) and an endocrine disruptor (3). Some studies revealed that the migration of DEHP from medical devices (4–6), childcare articles (7), and food packaging (8), resulted in critical problems in human health.

Yang et al. reported that oral administration of acetone-dissolved DEHP (0.2% containing diet) for 10 days to male C57BL/6 mice led to an obvious atrophy of the thymus and spleen (9). This result suggests that DEHP impairs the thymic differentiation process of T cells and the immunity in peripheral

tissues. Together with the issue of DEHP migration from medical devices in dialysis patients, the immunological effects of DEHP should be examined in detail.

In this present study, we examined the immunotoxicity induced by DEHP in accordance with the experimental design of Yang et al. (9). After dietary exposure for 10 or 20 days of corn oil-suspended DEHP (0.1% or 0.2% containing diet) to male C57BL/6 mice, the states of thymic differentiation (phenotypic proportion and surface expression of several molecules, CD3, CD4, CD8 and CD28, on T cells) and the mitogenesis of splenic T cells were analyzed. The results obtained in the present study contradict previous observations (9).

Materials and Methods

Chemicals and antibodies

The sources of the three main chemicals were as follows: DEHP from Aldrich Chemical, Milwaukee, WI; [³H]-thymidine from ICN Biomedicals, Irvine, CA; and corn oil from Hayashi Chemicals, Tokyo, Japan. The sources of the five monoclonal antibodies were as follows: 145-2C11 (hamster anti-CD3 ϵ) from Southern Biotechnology Associates, Birmingham, AL; CT-CD4 (rat anti-CD4) from Caltag Laboratories, San Francisco, CA; 5H10 (rat anti-CD8) from Caltag Laboratories; 2.4G2 (rat anti-CD16/32) from Pharmingen, San Diego, CA; 37.51 (hamster anti-CD28) from Southern Biotechnology Associates. Isotype

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control for each monoclonal antibody was purchased from Caltag Laboratories. Unless otherwise specified, all other chemicals were purchased from Sigma Chemicals Co., St. Louis, MO.

Animals and chemical exposure

Six-week-old male C57BL/6 mice (CLEA JAPAN, Tokyo, Japan) were housed in individual apartment cages in an air-conditioned room ($22\pm1^{\circ}\text{C}$ and $50\pm10\%$ humidity) with a 12-hr alternating light-dark cycle. The animals were given rodent chow and water *ad libitum* and allowed to acclimate for 1 week before treatment. The animals were subsequently divided into three groups. The mean body weights of each group were similar. DEHP was dissolved at 2% and 4% (w/w) in corn oil. The semipurified pellet diet for each treatment group was prepared using the DEHP-containing corn oil. The composition (w/w) of the diet was as follows: casein, 20.0%; DL-methionine, 0.3%; sucrose, 45%; corn starch 15%; mineral mix (AIN-76), 3.5%; vitamin mix (AIN-76), 1.0%; choline bitartrate, 0.2%; cellulose, 5.0%; and corn oil, 5.0%. The final dietary concentrations of DEHP were (w/w): 0% in the control group, 0.1% in the low group, and 0.2% in the high group. Animals were administered the control or treatment diet for 10 or 20 days. On termination of each treatment period, the mice were euthanized by CO_2 asphyxia.

Cell preparation and cell culture

The spleen, thymus and liver were surgically removed from male C57BL/6 mice. Each cell suspension of the spleen and thymus was obtained with RPMI-1640 medium by gently pushing these organs through a stainless steel mesh. Splenocytes were hemolysed with ammonium chloride. After washing thrice with medium, the cell viability was determined by the trypan blue exclusion test ($>97\%$). Splenocytes obtained were also applied through nylon wool columns to obtain splenic T cells (10). The purities of splenic T cells in cell suspensions were $>95\%$ $\text{CD3}\epsilon^{+}$ as assessed by cytometry (described below). Splenic T cells were cultured at 1×10^5 cells/well in 145-2C11- (5 $\mu\text{g}/\text{ml}$) with or without 37.51- (5 $\mu\text{g}/\text{ml}$) coated 96-well plates in HEPES buffered RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, 10% fetal bovine serum and antibiotics. After cultivation for 54 hr at 37°C in a 5% CO_2 atmosphere, each well was pulsed for 18 hr with 1 μCi of [^3H]-thymidine before cells were harvested. [^3H]-thymidine incorporation was measured using a scintillation counter.

Cytometry analysis

Splenocytes and thymocytes were resuspended in phosphate-buffered saline (PBS) containing 0.1% sodium azide. Each cell was then incubated with 2.4G2 for 5 min at 4°C to block nonspecific binding of fluorescein-conjugated mAb. Each cell was stained with fluorescein (fluorescein isothiocyanate or phycoerythrin)-conjugated mAbs. The stained cells were washed with PBS containing sodium azide, and resuspended in PBS supplemented with paraformaldehyde. After the exclusion of dead cells by forward and side light scattering, the phenotype and antigen expression were analyzed with cytometry (EPICs Elite; Coulter, Miami, FL) (11). The data from 20,000 cells

were subjected to calculate of the phenotypic proportion and expression of surface antigens. The expression of surface antigen was represented as relative fluorescence intensity obtained by subtracting the median intensity value for the cells stained with the isotype control.

Statistical analysis

Each group consisted of six mice. The values were expressed as means $\pm$ SEM. The significance of differences between values was analyzed using the one-factor analysis of variance followed by Fisher's Protected Least Significant Difference performed on Statview software (Cricket Software, Philadelphia, PA).

Results and Discussion

This study analyzed the contributions of DEHP to immunity. DEHP did not alter body weight gain, thymus weight, or spleen weight, although it induced hepatic hypertrophy (Table 1). This compound did not significant change the number of thymocytes and splenocytes (Table 2). These findings showed that DEHP did not cause any atrophy in the thymus or spleen. This conflicted with a recently published study (9), wherein mice fed on a 0.2% DEHP-containing diet for 10 days showed

Table 1 Effects of DEHP administration on body weight gain and tissue weight

	Control	Low	High
10 days treatment			
Body weight gain (g)	1.93 $\pm$ 0.28	2.13 $\pm$ 0.13	1.82 $\pm$ 0.15
Liver (g/100 g body weight)	5.72 $\pm$ 0.22 ^a	6.27 $\pm$ 0.07 ^b	7.81 $\pm$ 0.17 ^c
Thymus (mg/100 g body weight)	157 $\pm$ 8	152 $\pm$ 5	159 $\pm$ 7
Spleen (mg/100 g body weight)	265 $\pm$ 5	265 $\pm$ 5	257 $\pm$ 5
20 days treatment			
Body weight gain (g)	3.01 $\pm$ 0.34	3.19 $\pm$ 0.20	2.90 $\pm$ 0.44
Liver (g/100 g body weight)	5.60 $\pm$ 0.18 ^a	6.66 $\pm$ 0.15 ^b	7.45 $\pm$ 0.19 ^c
Thymus (mg/100 g body weight)	152 $\pm$ 8	148 $\pm$ 6	148 $\pm$ 9
Spleen (mg/100 g body weight)	271 $\pm$ 7	260 $\pm$ 3	266 $\pm$ 4

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 10 or 20 days. Values are means $\pm$ S.E. of six mice. Different superscript letters (a-c) in the same line denote significant differences ($p<0.05$).

Table 2 Effects of DEHP administration on the cell numbers of the thymus and spleen

	Control	Low	High
10 days treatment			
Thymocytes ($\times 10^8$)	1.22 $\pm$ 0.08	1.23 $\pm$ 0.10	1.24 $\pm$ 0.12
($\times 10^8/0.1$ g tissue)	3.58 $\pm$ 0.24	3.46 $\pm$ 0.27	3.35 $\pm$ 0.23
Splenocytes ($\times 10^7$)	9.62 $\pm$ 0.48	10.1 $\pm$ 0.6	9.07 $\pm$ 0.69
($\times 10^8/0.1$ g tissue)	1.53 $\pm$ 0.10	1.67 $\pm$ 0.09	1.48 $\pm$ 0.07
20 days treatment			
Thymocytes ($\times 10^8$)	1.16 $\pm$ 0.09	1.08 $\pm$ 0.04	1.18 $\pm$ 0.09
($\times 10^8/0.1$ g tissue)	2.97 $\pm$ 0.09	2.83 $\pm$ 0.10	3.13 $\pm$ 0.15
Splenocytes ($\times 10^7$)	9.17 $\pm$ 0.10	9.24 $\pm$ 0.36	9.62 $\pm$ 0.23
($\times 10^8/0.1$ g tissue)	1.32 $\pm$ 0.04	1.37 $\pm$ 0.06	1.43 $\pm$ 0.06

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 10 or 20 days. Values are means $\pm$ S.E. of six mice.

marked atrophy of the thymus and spleen, although the present study were employed experimental conditions similar to those of Yang et al. (9), such as strain of mice, level of DEHP and administration route. Although the atrophy in the thymus and spleen did not occur in the present study, the hepatic hypertrophy observed was in accord with previous findings (9). The hepatic hypertrophy in mice appears to be caused by absorption of DEHP as peroxisome proliferators (12–14). This suggests that the mice are significantly exposed to DEHP from the standpoint of DEHP treatments. Therefore, the present results suggested that DEHP elicits hypertrophy of the liver, but not atrophy of the thymus or spleen.

Although the reason for the different findings with regard to the weights of immune tissues (the thymus and spleen) is unclear, it may be due to differences in the preparation methods for the DEHP-containing diets. In the present study, we used corn oil as the vehicle for DEHP, while Yang et al. used acetone as that for DEHP (9); namely they removed the solvent by volatilization by leaving it more than 24 hr in a ventilated hood. It is known that in some cases combined exposure to multiple chemicals leads to additive or synergistic effects on physiologic states including immunity (15). Although further studies are needed to clarify this discrepancy, it may be based on the difference in the preparation method of the two DEHP diets.

To analyze the mechanism involved in DEHP immunotoxicity, a further study was conducted. The present study focused on whether DEHP exposure elicited alterations not only on the surface expression of these molecules but also on the proportion of the phenotype in intricately segregated thymic stages and in

Table 3 Effects of DEHP administration for 10 days on the expression of each surface molecule of T cells

Molecule	Phenotype	Control	Low	High
Thymus				
CD3	CD3 ^{low} CD4 ^{high}	1.33±0.02	1.36±0.04	1.38±0.04
	CD3 ^{high} CD4 ^{high}	16.6±0.4	16.6±0.3	15.9±0.5
	CD3 ^{low} CD8 ^{high}	1.39±0.06	1.34±0.03	1.37±0.02
	CD3 ^{high} CD8 ^{high}	13.2±0.3	13.4±0.3	13.3±0.4
CD4	CD4 ^{high} CD8 ^{high}	6.44±0.12	6.46±0.15	6.41±0.13
	CD4 ^{high} CD8 ^{low}	6.81±0.04	6.75±0.16	6.75±0.10
	CD4 ^{high} CD8 ⁻	10.9±0.2	10.9±0.3	10.7±0.2
CD8	CD4 ^{high} CD8 ^{high}	45.0±0.2	45.4±0.5	44.6±0.7
	CD4 ^{low} CD8 ^{high}	37.4±1.0	38.6±1.3	39.2±1.3
	CD4 ⁻ CD8 ^{high}	56.2±2.8	50.5±3.4	53.7±4.1
CD28	CD4 ^{low} CD28 ⁺	1.76±0.10	1.74±0.15	1.85±0.14
	CD4 ^{high} CD28 ⁺	1.61±0.12	1.62±0.18	1.68±0.16
	CD8 ^{low} CD28 ⁺	1.22±0.10	1.41±0.10	1.46±0.14
	CD8 ^{high} CD28 ⁺	1.40±0.14	1.66±0.14	1.69±0.24
Spleen				
CD3	CD3 ⁺ CD4 ⁺	19.3±0.2	19.0±0.3	18.7±0.5
	CD3 ⁺ CD8 ⁺	13.9±0.1	13.8±0.2	14.1±0.2
CD4	CD4 ⁺ CD8 ⁻	14.5±0.2	14.5±0.2	14.2±0.5
CD8	CD4 ⁻ CD8 ⁺	40.4±1.0	41.2±0.7	38.5±1.7
CD28	CD4 ⁺ CD28 ⁺	0.537±0.074	0.548±0.028	0.565±0.037
	CD8 ⁺ CD28 ⁺	0.395±0.016	0.395±0.031	0.405±0.034

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 10 days. Fluorescence intensity is represented as the relative intensity as described in the Materials and Methods section. Values are means±S.E. of six mice.

splenic T cells. There was no change in the states of T cells characterized by both the expression of all surface molecules and the phenotypic composition (Tables 3–6). Furthermore, the present DEHP exposures did not affect the mitogenesis of splenic T cells, regardless of the existence of the stimulation mediated by a costimulatory signal (Fig. 1). The differentiation and maturation stages of T cells in the thymus are characterized by the composition of multiple phenotype expressing surface

Table 4 Effects of DEHP administration for 20 days on the expression of each surface molecule of T cells

Molecule	Phenotype	Control	Low	High
Thymus				
CD3	CD3 ^{low} CD4 ^{high}	1.55±0.07	1.46±0.05	1.38±0.04
	CD3 ^{high} CD4 ^{high}	17.1±0.2	17.4±0.3	17.1±0.3
	CD3 ^{low} CD8 ^{high}	1.60±0.07	1.47±0.07	1.48±0.02
	CD3 ^{high} CD8 ^{high}	13.5±0.3	13.3±0.3	13.0±0.2
CD4	CD4 ^{high} CD8 ^{high}	6.59±0.09	6.56±0.08	6.41±0.11
	CD4 ^{high} CD8 ^{low}	6.40±0.05	6.52±0.06	6.71±0.14
	CD4 ^{high} CD8 ⁻	10.4±0.2	10.3±0.2	10.7±0.2
CD8	CD4 ^{high} CD8 ^{high}	50.2±1.5	50.7±2.0	50.3±0.9
	CD4 ^{low} CD8 ^{high}	38.5±1.8	39.6±1.5	39.8±0.6
	CD4 ⁻ CD8 ^{high}	57.8±1.7	58.1±0.4	58.2±0.9
CD28	CD4 ^{low} CD28 ⁺	1.08±0.02	1.08±0.05	1.10±0.04
	CD4 ^{high} CD28 ⁺	1.01±0.02	0.940±0.043	0.927±0.039
	CD8 ^{low} CD28 ⁺	0.793±0.029	0.775±0.021	0.868±0.045
	CD8 ^{high} CD28 ⁺	0.843±0.062	0.805±0.082	0.835±0.101
Spleen				
CD3	CD3 ⁺ CD4 ⁺	20.2±0.2	19.9±0.2	20.6±0.5
	CD3 ⁺ CD8 ⁺	15.1±0.1	14.8±0.1	15.4±0.3
CD4	CD4 ⁺ CD8 ⁻	14.4±0.2	14.4±0.1	14.4±0.2
CD8	CD4 ⁻ CD8 ⁺	35.4±0.7	35.2±0.8	34.2±0.3
CD28	CD4 ⁺ CD28 ⁺	0.294±0.021	0.291±0.026	0.287±0.021
	CD8 ⁺ CD28 ⁺	0.265±0.022	0.236±0.017	0.239±0.017

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 20 days. Fluorescence intensity is represented as the relative intensity as described in the Materials and Methods section. Values are means±S.E. of six mice.

Table 5 Effects of DEHP administration for 10 days on proportions of each phenotype expressing surface molecules of T cells

Phenotype	Control	Low	High
Thymus			
CD3 ⁻ CD4 ^{high}	20.2±2.0	23.3±2.1	24.7±1.0
CD3 ^{low} CD4 ^{high}	58.5±0.8	58.6±1.3	61.4±1.4
CD3 ^{high} CD4 ^{high}	8.4±0.2	9.0±0.4	8.5±0.4
CD3 ⁻ CD8 ^{high}	24.4±1.0	24.7±1.8	22.0±1.0
CD3 ^{low} CD8 ^{high}	55.9±1.7	60.2±2.0	62.5±2.7
CD3 ^{high} CD8 ^{high}	2.4±0.1	2.8±0.2	2.7±0.2
CD4 ⁻ CD8 ⁻	0.57±0.04	0.45±0.06	0.45±0.06
CD4 ⁺ CD8 ⁺	88.5±0.4	89.0±0.3	89.6±0.6
CD4 ⁺ CD8 ⁻	8.3±0.5	7.8±0.4	7.3±0.7
CD4 ⁻ CD8 ⁺	2.6±0.2	2.7±0.3	2.7±0.2
Spleen			
CD3 ⁺	64.1±1.9	66.3±3.9	64.7±5.1
CD4 ⁺ CD8 ⁻	42.3±1.9	42.9±2.7	38.9±1.4
CD4 ⁻ CD8 ⁺	25.1±1.5	24.1±2.4	24.1±2.4

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 10 days. Values are means±S.E. of six mice.

Table 6 Effects of DEHP administration for 20 days on proportions of each phenotype expressing surface molecules of T cells

Phenotype	Control	Low	High
Thymus			
CD3 ⁺ CD4 ^{high}	12.6±2.7	12.9±2.0	16.1±2.8
CD3 ^{low} CD4 ^{high}	70.6±1.4	73.0±1.6	71.5±2.4
CD3 ^{high} CD4 ^{high}	10.5±0.3	9.5±0.6	9.5±0.3
CD3 ⁺ CD8 ^{high}	10.4±1.4	13.7±2.0	11.8±1.4
CD3 ^{low} CD8 ^{high}	73.3±0.8	74.2±1.2	76.5±1.5
CD3 ^{high} CD8 ^{high}	3.0±0.1	2.7±0.2	2.7±0.1
CD4 ⁺ CD8 ⁺	0.75±0.09	0.77±0.08	0.73±0.06
CD4 ⁺ CD8 ⁺	88.5±0.5	89.9±0.4	89.7±0.5
CD4 ⁺ CD8 ⁺	6.8±0.4	6.1±0.3	6.3±0.4
CD4 ⁺ CD8 ⁺	3.7±0.2	3.3±0.1	3.2±0.1
Spleen			
CD3 ⁺	48.8±1.5	45.0±2.3	48.3±1.8
CD4 ⁺ CD8 ⁺	32.7±0.9	31.0±1.0	32.9±1.6
CD4 ⁺ CD8 ⁺	7.4±0.9	7.0±0.9	6.9±0.1

Male C57BL/6 mice were administered either a control, 0.1% (Low) or 0.2% (High) DEHP-containing diet for 20 days. Values are means±S.E. of six mice.

molecules, including CD3, CD4, CD8 and CD28 (16–18). In addition to the indispensability of these molecules in thymic differentiation, they play pivotal roles in recognizing antigens associated with the major histocompatibility complex expressed on antigen-presenting cells (19). This recognition subsequently leads to signals for clonal expansion of T cells. The primary signal mediated by CD3 and CD4 (or CD8) requires at least one more concurrent signal mediated by some costimulatory molecules, such as CD28 (19). Therefore, both the phenotypic composition and the states of surface expression of these molecules (CD3, CD4, CD8, and CD28) have important implications for the dynamics of T cells. Taken together, the present observations demonstrate that DEHP does not disrupt either thymic maturation

Fig. 1 Effects of DEHP administration for 10 days (left) or 20 days (right) on mitogenesis of splenic T cells. Cell culture plates were coated with 145-2C11 (hamster anti-CD3ε), 37.51 (hamster anti-CD28) and/or control immunoglobulin. Splenic T cells were cultured with indicated monoclonal antibody at a density of 1×10^5 cells/well as described in the Materials and Methods section. Values are the means±S.E. of six mice.

tion or antigen-induced clonal expansion of splenic T cells.

Recent *in vitro* studies suggested that DEHP and its hydrolysis products regulate inflammatory functions of leukocytes, including mast cells (20), neutrophils (21), monocytes (22), and polymorphonuclear blood cells (22, 23). Although the other reports (15, 20–23) warrant further *in vivo* investigations to elucidate both the contribution of DEHP on the inflammation and the modulatory effects caused by coadministration of some other chemicals, DEHP does not serve as an immunoregulator by suppressing the functions of T cells under the short-term exposure conditions to mice.

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