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Di-(2-ethylhexyl) phthalate affects immune cells from atopic prone mice *in vitro*

Eiko Koike*, Ken-ichiro Inoue, Rie Yanagisawa, Hirohisa Takano*

Environmental Health Sciences Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305-8506, Japan

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ABSTRACT

Phthalate esters as plasticizers have been widespread in the environment and may be associated with development of allergic diseases such as asthma and atopic dermatitis. However, the underlying mechanisms have not been fully elucidated. The present study investigated the effects of di-(2-ethylhexyl) phthalate (DEHP) on immune cells from atopic prone NC/Nga mice *in vitro*. Bone marrow-derived dendritic cells (BMDC) as a professional antigen-presenting cell and splenocytes as mixture of immune cells were used. BMDC were differentiated by culture with granulocyte macrophage-colony stimulating factor (GM-CSF) in the presence of DEHP (0.1–10 μM) for 6 days. In another experiments, BMDC were differentiated by culture with GM-CSF for 8 days then these BMDC were exposed to DEHP (0.1–100 μM) for 24 h. Splenocytes were exposed to DEHP for 24 h (0.1–100 μM) or 72 h (0.1–1000 nM). After the culture, the phenotypic markers and the function of BMDC and splenocytes were evaluated. BMDC differentiated in the presence of DEHP showed enhancement in the expression of MHC class II, CD86, CD11c and DEC205, and in their antigen-presenting activity. On the other hand, the function of the differentiated BMDC was not activated by DEHP although DEHP partly enhanced their expression of DEC205. DEHP-exposed splenocytes showed increases in their TCR and CD3 expression, interleukin-4 production, and antigen-stimulated proliferation. These results demonstrate that DEHP enhances BMDC differentiation but not activation and also enhances Th2 response in splenocytes from atopic prone mice. The enhancement might contribute to the aggravating effect of DEHP on allergic disorders.

1. Introduction

Phthalate esters, ubiquitously used as plasticizers, have been widespread in the environment (Wormuth et al., 2006). The total global consumption of phthalate esters is estimated to exceed 3.5 million metric tons/year, with di-(2-ethylhexyl) phthalate (DEHP) constituting approximately 50% of the consumption (Cadogan and Howick, 1996). DEHP is used in manufacturing of articles made of polyvinyl chloride, which is used in consumer products, flooring and wall coverings, food contact applications, and medical devices (Calafat and McKee, 2006). The route of human exposure to DEHP is ingestion, inhalation, and dermal contact. It has been reported that serum DEHP concentration of healthy volunteer is less than 1 μg/ml (2.56 μM) (Latini, 2000; Luisi et al., 2006).

Several epidemiological studies have suggested that exposure to phthalate esters may be associated with development of asthma, wheezing, and allergic symptoms (Bornehag et al., 2004; Jaakkola et al., 2006, 1999, 2004). The study by Bornehag et al. has revealed the positive association between allergic asthma in children and phthalate esters in house dust.

On the other hand, experimental reports have indicated that DEHP can act as an adjuvant to stimulate antigen-specific immunoglobulin production (Larsen et al., 2002; Thor Larsen et al., 2001). In our previous study, DEHP has enhanced atopic dermatitis-like skin lesions in atopic prone NC/Nga mice at 100-fold lower levels than the no observed adverse effect level determined on histological changes in the liver of rodents (Takano et al., 2006; Yanagisawa et al., 2008). Furthermore, the enhancing effects of DEHP have been paralleled to the infiltration of eosinophils, mast cell degranulation, and the expression of proinflammatory molecules such as eotaxin and macrophage inflammatory protein-1α in the inflamed skin. However, the mechanisms via which DEHP exacerbates the atopic dermatitis have not been fully elucidated.

In general, activation of antigen presentation can lead to an amplification of the antigen-related immunoglobulin production and allergic inflammation through the proliferation/activation of lymphocytes and eosinophils (van Rijt and Lambrecht, 2001; Wills-Karp, 1999). Therefore, antigen-presenting cells (APC) and lymphocytes should play an important role in the possible mechanisms of the exacerbation of allergic/atopic diseases and/or responses. Dendritic cells (DC), as professional APC, are the most

* Corresponding authors. Tel.: +81 29 850 2336; fax: +81 29 850 2334.
E-mail addresses: ekoike@nies.go.jp (E. Koike), inoue.kenichirou@nies.go.jp (K.-i. Inoue), yanagi@nies.go.jp (R. Yanagisawa), htakano@nies.go.jp (H. Takano).

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capable inducers not only in the initiation of primary immune responses but also in the promotion of secondary immune responses. MHC class II molecules (Niederhuber and Shreffler, 1977) and co-stimulatory molecules such as CD80 and CD86 (Freeman et al., 1993; Lenschow et al., 1993) are essential for antigen presentation. As well, DC maturation/activation is defined by enhanced expression of MHC class II and co-stimulatory molecules and enhanced antigen-presenting activity determined by increased T-cell proliferation and amplified cytokine production (Banchereau and Steinman, 1998; Thomas and Lipsky, 1996). On the other hand, the splenocytes are mixture of T cells, B cells, and APC and play an important role in the general immune responses and/or diseases. Accordingly, alteration of phenotypes and cytokine production of splenocytes may also affect allergic/atopic diseases and/or responses.

In the present study, we investigated whether DEHP affects the immune cells from atopic prone mice *in vitro* using bone marrow-derived DC (BMDC) and splenocytes from NC/Nga mice.

2. Materials and methods

2.1. Animals

Seven-week-old SPF NC/NgaTndCrlj male mice were purchased from Charles River Japan (Osaka, Japan) and were used at 11–15-week old (weighing 24–27 g). Mice were given sterile distilled water and a commercial diet (CE-2; CLEA Japan Inc., Tokyo, Japan) *ad libitum*. They were housed in an animal facility that was maintained at 24–26 °C with 55–75% humidity and a 12-h light/dark cycle under conventional conditions. The procedures of all animal studies were approved by the Institutional Review Board of National Institute for Environmental Studies.

2.2. Preparation of bone marrow cells and splenocytes

Mice were anesthetized with sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) given intraperitoneally (50 mg/kg), and exsanguinated from the cut abdominal aorta and vein. After removing the surrounding muscle tissue, the bones were left in 70% ethanol for 3 min and washed with Dulbecco's calcium and magnesium-free, phosphate-buffered saline (PBS; Takara Bio Inc., Shiga, Japan). Both ends of the bones were cut and then the marrow was flushed with PBS using a syringe with 25G needle. The marrow suspension was passed through sterile nylon mesh to remove small pieces of bone and debris and red blood cells were lysed with ammonium chloride. Spleen was pushed through a sterile 200 mesh stainless steel sheet and red blood cells were also lysed with ammonium chloride. The cells were centrifuged at 400 × *g* for 5 min at 20 °C. After washing twice with PBS, the cells were resuspended in culture medium R10, which was GIBCO RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; MP Biomedicals Inc., Eschwege, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 50 μM 2-mercaptoethanol (Invitrogen). The numbers of viable cells were determined by the trypan blue (Invitrogen) exclusion method.

2.3. Differentiation of BMDC

BMDC were differentiated using a modified protocol of Lutz et al. (1999). In the first experiment, bone marrow cells (4×10^5 ml^{-1}) were cultured in R10 medium containing 10 ng/ml recombinant mouse GM-CSF (Sigma) at 37 °C in a 5% CO_2/95% air atmosphere. At day 3, another same volume of the medium containing 10 ng/ml GM-CSF was added to the culture. At day 6, non-adherent and loosely adherent cells were collected by gentle pipetting. In the second experiment, bone marrow cells (4×10^5 ml^{-1}) were cultured in R10 medium containing 20 ng/ml GM-CSF. At day 3, another same volume of the medium containing 20 ng/ml GM-CSF was added to the culture. At day 6, half the culture medium was replaced with fresh medium. At day 8, non-adherent and loosely adherent cells were collected by gentle pipetting. The differentiated BMDC were centrifuged at 400 × *g* for 5 min at 20 °C and were resuspended in fresh medium. The numbers of viable cells were determined by the trypan blue exclusion method.

2.4. Exposure to DEHP

DEHP (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO; Sigma) and was sonicated for 3 min using an ultrasonic disrupter (UD-201; TOMY, Tokyo, Japan). BMDC were differentiated by culture with GM-CSF as described above. In the first experiment, bone marrow cells (4×10^5 ml^{-1}) were exposed to DEHP (0.1–10 μM) or 0.1% DMSO (control) with GM-CSF (10 ng/ml) for 6 days to investigate the effects of DEHP on the differentiation of BMDC. In the second experiment, the differentiated BMDC (1×10^6 ml^{-1}) at day 8 of culture with GM-CSF (20 ng/ml) were exposed to DEHP (0.1–100 μM) or 0.1% DMSO (control) in the presence of GM-CSF (10 ng/ml) for 24 h to investigate the effects of DEHP on the activation of the differentiated BMDC. Thereafter, their phenotypes and their function in terms of antigen-presenting activity and cytokine production from responder T cells were evaluated. On the other hand, splenocytes (1×10^6 ml^{-1}) were exposed to DEHP (0.1–100 μM) or 0.1% DMSO (control) for 24 h and then their phenotypes and cytokine production were examined. Moreover, splenocytes (1×10^6 ml^{-1}) were exposed to DEHP (0.1–1000 nM) or 0.1% DMSO (control) in the presence of 10 μg/ml mite extract [*Dermatophagoides pteronyssinus* crude extract (Dp); Cosmo Bio LSL, Tokyo, Japan] for 72 h and then their antigen-stimulated lymphocyte proliferation was measured. Each experiment was performed three individual cultures from three animals for each DEHP concentration and three independent experiments were done.

2.5. FACS analysis

For FACS analysis, the following monoclonal antibodies were used: MHC class II molecules: I-A/I-E (2G9, FITC-conjugated, BD Biosciences Pharmingen, CA); co-stimulatory molecules: CD80 (16-10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen); DC markers: CD11c (HL3, PE-conjugated, BD Biosciences Pharmingen), DEC205 (NLDC-145, PE-conjugated, Miltenyi Biotech GmbH, Gladbach Germany); B-cell marker: CD19 (1D3, BD Biosciences Pharmingen); T-cell markers: TCR β chain (H57-597, BD Biosciences Pharmingen), CD3 complex (17A2, BD Biosciences Pharmingen); co-stimulatory receptor: CD28 (37.51, BD Biosciences Pharmingen); interleukin (IL)-4 receptor (IL-4R): CD124 (mIL4R-M1, BD Biosciences Pharmingen). After DEHP exposure, the cells ($3–5 \times 10^5$) were resuspended in 100 μl PBS with 0.3% bovine serum albumin and 0.05% sodium azide (Wako Pure Chemical Industries) and were incubated with 1 μg amount of each antibody for 30 min on ice. After incubation, the cells were washed, and the fluorescence was measured by a FACSCalibur (Becton, Dickinson and Company, NJ). For each sample, fluorescence data from 10,000 cells were collected and positive cells were expressed % total events.

2.6. Evaluation of BMDC function

BMDC function was evaluated by their antigen-presenting activity and their stimulating capacity for cytokine production from responder T cells. DEHP-exposed BMDC were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) for 20 min in a water bath at 37 °C. Responder T cells were derived from a pool of splenocytes from antigen-sensitized NC/Nga mice (three mice/each experiment). Briefly, mice were immunized with 50 μg Dp and 1 mg $Al(OH)_3$ in 0.1 ml of saline given intraperitoneally and splenocytes were harvested at day 14. T cells were isolated from the splenocytes using a nylon fiber column (Wako Pure Chemical Industries). Thereafter, Dp-sensitized T cells (2×10^5) were co-cultured with BMDC (from 2.5×10^3 to 1×10^4) in the presence of 2 μg of Dp in 200 μl of R10 medium in 96-well flat-bottom plates. Co-culture of BMDC and T cells was performed in triplicate at 37 °C in a 5% CO_2/95% air atmosphere. After 91 h, T-cell proliferation as an indicator of antigen-presenting activity and cytokine production from T cells were measured.

2.7. Measurement of cell proliferation

Cell proliferation was measured with a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. This technique is based on the incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. BrdU incorporated into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-BrdU antibodies. Cell proliferation was measured by adding BrdU to each well 20 h before the measurement. Absorbance of the samples was measured in an ELISA reader at a wavelength of 450 nm.

2.8. Measurement of cytokines

Levels of interferon (IFN)-γ (Endogen, Cambridge, MA), IL-4 (Amersham, Buckinghamshire, UK), and IL-10 (Endogen) in culture supernatant were measured by using ELISA according to the manufacturer's instructions. The detection limit of IFN-γ, IL-4, or IL-10 was 10, 5, 12 pg/ml, respectively.

2.9. Statistical analysis

Data were represented as the mean ± S.E.M. of three individual culture from three animals per each experiment. The analysis of the effects of independent variables of DEHP concentration was done by ANOVA with Fisher's LSD test. A p value of <0.05 was considered to indicate a significant difference.

3. Results

3.1. Effects of DEHP on the differentiation of BMDC

Bone marrow cells were cultured with GM-CSF in the presence or absence of DEHP for 6 days. Then, the expression of cell surface molecules and antigen-presenting activity of the BMDC were