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Di-(2-ethylhexyl) phthalate adjuvantly induces imbalanced humoral immunity in ovalbumin-sensitized BALB/c mice ascribing to T follicular helper cells hyperfunction

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) has been considered as a widespread environmental persistent organic pollutant and its potential concern on human health is raised by previous studies. In particular, children are more likely to be exposed to DEHP through gastrointestinal route and consequently are more susceptible to DEHP hazards. Some reports have uncovered a positive association between DEHP exposure and an increased prevalence of allergic diseases in infants and juveniles. Allergy is a hypersensitive reaction rooted in imbalanced humoral immunity. T follicular helper cell (Tfh), an important $CD4^+$ Th cell subset, until recently has been identified as a key player in humoral immune response by modifying B cells functions. Tfh cells are therefore perceived as the therapeutic target of immune disorders. In the present study, focusing on the newly confirmed Tfh cells, we examined the effects of DEHP on humoral immunity and investigated the underlying mechanisms. Using ovalbumin (OVA) sensitization weanling mice model under the condition of gastrointestinal exposure to DEHP, we found that DEHP acted as an immunoadjuvant to augment OVA-specific IgE and IgG1 production, amplified germinal center formation in lymphoid nodule, as well as stimulated the expansion of $CD4^+CXCR5^+ICOS^+$/$CD4^+CXCR5^+PD\text{-}1^+$ Tfh cells and $CD19^+CD138^+GL7^+$ plasma cells. Based on the results of immune adoptive transfusion, DEHP-related anaphylactic response was ascribed to Tfh cells hyperfunction directly. We further proved that DEHP gavage together with OVA sensitization adjuvantly promoted the synthesis of cytokines IL-21 and IL-4 and the expression of transcription factors Bcl-6 and c-Maf in Tfh cells. In conclusion, our study demonstrates that DEHP has adjuvant toxic effects on Tfh cells by synthesizing an excess of cytokines IL-21 and IL-4 via over-expression of transcription factors Bcl-6 and c-Maf, leading to an increasing secretion of allergy-related IgE and IgG1.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate plasticizer for imparting flexibility to polyvinyl chloride (PVC) formulations. It is commonly applied in food packaging, children's toys, building materials, medical devices, and personal care products. Due to its widely application and chemical structure stability, together with the characteristics of its non-covalently binding to and easily leaching out from PVC, DEHP is frequently detected in environmental mediums such as soil, water and atmosphere dust. It has been considered as a widespread environmental persistent organic pollutant and its potential concern on human health is highlighted on a global scale (Chen et al., 2012; North and Halden, 2013). Though human exposure routes to DEHP are multiple, including diet, inhalation, skin and medical contact, ingestion of contaminated foods, water and other materials is thought to be a major route. Once DEHP is absorbed into digestive tract, it can enter hepatoenteral circulation and be rapidly metabolized into the primary product mono-2-ethylhexyl

Abbreviations: DEHP, di-(2-ethylhexyl) phthalate; MEHP, mono-2-ethylhexyl phthalate; OVA, ovalbumin; PMA, phorbol myristate acetate; PNA, peanut agglutinin; PVC, polyvinyl chloride; PVDF, polyvinylidene difluoride; SCID, severe combined immune deficient; Tfh, T follicular helper cell.

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phthalate (MEHP) together with a number of secondary products in the liver (Hayashi et al., 2012; Lorber and Calafat, 2012). DEHP reference dose (RfD) of U.S. Environmental Protection Agency (EPA) is 20 μg/kg each day, and the tolerable daily intake (TDI) of European Union is 20–48 μg/kg. Agency for Toxic Substances and Disease Registry (ATSDR) has reported the daily intake range of DEHP for an adult is estimated to be 3–30 μg/kg. In particular, children's daily exposure assessment is up to 85 μg/kg as they are more likely to be exposed to DEHP by sucking or chewing on plastic products such as bottles and toys. Thus DEHP becomes a heavily pollution source especially among children (Braun et al., 2013; Chang et al., 2014).

Recent epidemiological investigations show that the occurrence rate of hypersensitive diseases including allergic rhinitis, bronchial asthma and atopic dermatitis has increased rapidly, especially in infants and juveniles, which is believed to be related to an increased production of phthalate plasticizers. DEHP has been reported to enhance the potency of anaphylaxis and thereby plays an important role in the development of allergic diseases (Callesen et al., 2014; Hsu et al., 2012; Wang et al., 2014). The experimental evidences indicate that DEHP can disrupt the immune system through the modulation of cellular proliferation, lymphocytes division and cytokines synthesis (Kimber and Dearman, 2010). In some cases, these immune parameters show a relatively higher sensitivity in juvenile compared to adult animals (Tonk et al., 2012). Moreover, DEHP can also act as an immunoadjuvant to induce the improper enhancement of humoral immune response through various exposure routes (Guo et al., 2012; Larsen et al., 2007; Matsuda et al., 2010; Sadakane et al., 2014). The fundamental property of humoral immunity is B cell response supported by T helper cells (Th), principally including germinal center formation, plasma cells (antibody-forming cells) differentiation, immunoglobulins affinity maturation and class switch. Of particular importance is that B cell response depends on the assistance provided by Th cells (Gatto and Brink, 2010; Ramiscal and Vinuesa, 2013; Shlomchik and Weisel, 2012). Among Th cells, Th2 cell subset was once considered as a critical subpopulation to help B cells mediate antibody production, so most researchers over the past decade attempted to explain DEHP adjuvant toxic effects on exacerbating allergy through up-regulated cytokine signaling of Th2 cells. However, the explicit mechanisms have not been fully elucidated hitherto (Bornehag and Nanberg, 2010; Veldhoen, 2009). In order to find out rational treatment and preventive strategy of DEHP-related allergic diseases, it is necessary to carry out further study for expounding relevant mechanisms.

More recently, it has been found that Th2 cell-related cytokines (IL-4, IL-5 and IL-6) deficient mice can still generate antibody specific to thymus-dependent antigen, indicating that Th2 cell subset is not dominant support for B cells in humoral immune response (Kopf et al., 1995). Besides Th2 cells, a new subset of $CD4^+$ Th cells termed T follicular helper cell (Tfh) has been identified as the most important subpopulation that potently assists B cells to modulate antibody-mediated humoral immunity in germinal center (Johnston et al., 2009; Nurieva et al., 2009). Tfh cells retain intense expression of chemokine CXCR5, which functionally directs them toward B cells follicular area by the chemotaxis of specific receptor CXCR13 (Elsner et al., 2012). Tfh cells also express high level of some costimulatory molecules (ICOS and PD-1) that can specially bind to corresponding B cell surface ligands (ICOSL and PDL1/PDL2), accomplishing Tfh-B cell co-localization and interaction (Choi et al., 2011; Hams et al., 2011). And Tfh cells synthesize characteristic cytokines (IL-21 and IL-4), facilitating antibody production and class switch recombination (Karnowski et al., 2012; Luthje et al., 2012). Furthermore, the expression profile of typical transcription factors (Bcl-6 and c-Maf), along with their mutual regulation, is of crucial significance for initial differentiation and essential functions of Tfh cells (Kroenke et al., 2012; Liu et al., 2012).

In the present study, focusing on the newly confirmed Tfh cells, we examined the effects of DEHP on humoral immunity and investigated the underlying mechanisms using ovalbumin (OVA) sensitization weanling mice model under the condition of gastrointestinal exposure to DEHP. To our knowledge, this is the first time to ascertain DEPH immunotoxicity effects and the related mechanisms through the new perspective of Tfh cells.

2. Materials and methods

2.1. Chemicals

DEHP (CAS: 117-81-7, purity ≥ 99.5%), OVA (CAS: 9006-59-1, grade VII, purity ≥ 98%), phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Rhodamine-labeled peanut agglutinin (PNA) was purchased from Vector (Burlingame, California, USA). Additional sources and vendors of specific reagents, such as antibodies and kits, are listed below.

2.2. Animals

Since children are more susceptible to DEHP hazards, weanling mice were used in this animal study. BALB/c mice (2–3 weeks old, 10–12 g) and T/B lymphocytes severe combined immune deficient (SCID) mice (5–7 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Nantong University. Mice were fed with OVA-free food and distilled water ad libitum, and housed in a temperature-controlled (24–26 °C) room at 60 ± 5% humidity under a 12-h light/dark cycle. They were quarantined for at least 7 days before study initiation. All the experiments involving mice were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the Chinese National Committee for the Use of Experimental Animals for Medical Purposes, Jiangsu Province. All efforts were made to minimize the number of mice used with regard for alleviation of suffering.

2.3. Exposure and immunization protocols

Since gastrointestinal exposure is best accord with human actual exposure route, the murine model of DEHP gavage with OVA sensitization was established in this research. BALB/c mice were randomly divided into eight groups consisting of DEHP (30, 300, 3000 μg/kg) or corn oil gavage with OVA sensitization and DEHP (30, 300, 3000 μg/kg) or corn oil gavage with normal saline sham-sensitization. There are eight mice in each group, four male and four female. DEHP gavage dosage was chosen based on the current risk assessment parameters and safety limit values. DEHP was dissolved well in laboratory-grade corn oil at different concentrations for administration. All mice were gavaged with DEHP (30, 300, 3000 μg/kg) or corn oil at equivalent volume once a day from day 1 to 28 (28 times), and were sensitized with OVA (100 μg in 50 μl normal saline) by double subcutaneous injection into footpad on day 7 and 16. DEHP-only and vehicle-only control groups were sham-sensitized with 50 μl normal saline at the same time.

2.4. Quantification of serum OVA-specific immunoglobulins by enzyme-linked immunosorbent assay (ELISA)

Whole blood from different groups of BALB/c mice was drawn by heart puncture after finishing of the exposure and immunization treatments. Serum samples were collected by blood