

Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl)phthalate and ovalbumin in BALB/c mice

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Abstract

Background: Epidemiological studies have suggested an association between exposure to phthalate plasticizers, including di-(2-ethylhexyl)phthalate (DEHP), and increased prevalence of asthma, rhinitis or wheezing. Furthermore, studies in mice have demonstrated an adjuvant effect from DEHP after parenteral administration with the model allergen ovalbumin (OVA).

Objective: Exposures to DEHP were investigated for adjuvant effects and airway inflammation in a mouse inhalation model.

Methods: BALB/cJ mice were exposed to aerosols of 0.022–13 mg/m³ DEHP and 0.14 mg/m³ OVA 5 days/week for 2 weeks and thereafter weekly for 12 weeks. Mice exposed to OVA alone or OVA + Al(OH)₃ served as control groups. Finally, all groups were exposed to a nebulized 1% OVA solution on three consecutive days. Serum, bronchoalveolar lavage (BAL) fluid, and draining lymph nodes were collected 24 h later.

Results: In the OVA + Al(OH)₃ group, significantly increased levels of OVA-specific IgE and IgG1 in serum as well as of eosinophils in BAL fluid were observed. DEHP affected OVA-specific IgG1 production in a concentration-dependent manner, whereas little effect was seen on IgE and IgG2a. Dose-dependent increases in inflammatory cells were observed in BAL fluids, leading to significantly higher lymphocyte, neutrophil and eosinophil numbers in the OVA + 13 mg/m³ DEHP group. *Ex vivo* cytokine secretion by cultures of draining lymph nodes suggested that DEHP has a mixed Th1/Th2 cytokine profile.

Conclusion: Airborne DEHP is able to increase serum IgG1 and lung inflammatory cell levels, but only at very high concentrations. Realistic DEHP levels do not have an adjuvant effect or induce allergic lung inflammation in the present mouse model.

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1. Introduction

Numerous studies have proposed that environmental chemicals with adjuvant effect may contribute to the increasing number of allergic subjects in Westernized countries (e.g. Nielsen et al., *in press*). Phthalate plasticizers are widespread in the environment (Wormuth et al., 2006) and it has been shown that phthalates may

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have an effect on the immune system. With respect to human exposures, the phthalate di-(2-ethylhexyl) phthalate (DEHP) is interesting, as DEHP accounts for approximately 50% of the total global phthalate consumption of about 3.5 million metric tonnes (Cadogan and Howick, 1996). Phthalates are not antigenic in themselves (Butala et al., 2004) but animal studies have shown that certain phthalate plasticizers are immuno-modulatory by increasing the immunogenicity of a co-administered antigen (Larsen et al., 2001, 2002). Among the phthalates studied, DEHP has proved to be one of the most potent enhancers of immune responses to simultaneously administered ovalbumin (OVA) (Larsen et al., 2002). Furthermore, it was recently demonstrated that some phthalates, including DEHP, could enhance the release of histamine from isolated human basophils (Glue et al., 2005). The authors concluded that this effect may contribute to allergy symptoms in the patient.

Additionally, several epidemiological studies have suggested that phthalate plasticizers may play a role in the development of wheezing, rhinitis, asthma or asthma symptoms (Bornehag et al., 2004; Jaakkola et al., 1999, 2004, 2006). However, only the study by Bornehag et al. (2004) actually measured the DEHP levels. Even in this case the study furnishes little information about cause–effect relationships due to its cross-sectional design (World Health Organisation, 1983). Thus, controlled studies are necessary to avoid confounders. In the present study, we firstly performed acute airway exposures to DEHP to assess irritant and inflammatory effects which may promote adjuvant activity (Granum and Lovik, 2002). Secondly, we investigated the effect of long-term exposure to aerosolized DEHP and ovalbumin (OVA) on the immune response in mice. The assessment of effects on the immune response was based on levels of OVA-specific IgE, IgG1 and IgG2a antibodies, cell composition in bronchoalveolar lavage (BAL) fluid, and the cytokine profile of local lymph node cell cultures following restimulation with OVA *ex vivo*. Finally, a margin-of-exposure was estimated to address the relevance of DEHP exposure in humans.

2. Materials and methods

2.1. Animals

Inbred female BALB/cJ mice aged 5–6 weeks were purchased from Taconic M&B, Ry, Denmark, and were housed in polypropylene cages (380 mm × 220 mm × 150 mm) with pinewood sawdust bedding (Lignocel S8, Brogaarden, Den-

mark). The cages, each housing up to 10 mice, were furnished with bedding materials, gnaw sticks and cardboard tubes. The photo-period was from 6 a.m. to 6 p.m., and the temperature and mean relative humidity in the animal room were 19–22 °C and 43 ± 8% (S.D.), respectively. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, Germany) and tap water were available *ad libitum*. The mice used in the long-term exposure study were conditioned to experimental procedures by being placed in body (head-out) plethysmographs for 15 min daily on 5 consecutive days, 1 week before starting exposures. Body weights were monitored as a general health indicator and at the end of the experiment, the relative liver weights were determined as an indicator of systemic peroxisome proliferator-activated receptor (PPAR)- α activation. Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark.

2.2. Chemicals

DEHP (CAS 117-81-7, purity $\geq 97.0\%$) and acetone (CAS 67-64-1, purity $\geq 99.0\%$) were purchased from Fluka, Buchs, Germany. Chicken egg OVA (CAS 9006-59-1) was grade V (purity $\geq 98\%$) from Sigma–Aldrich, St. Louis, MO, USA. The Al(OH)₃ adjuvant was from Alhydrogel, Brenntag Biosector, Denmark. Calibration standard curves and solvents were as previously described (Clausen et al., 2004).

2.3. Endotoxin measurements

DEHP and OVA were assayed for content of endotoxins. A 10% (w/v) solution of DEHP was prepared in ethanol (96%). To isolate endotoxin, 5 mL of this solution was ultra filtered through an Ultrasart D20 (Sartorius, Edgewood, New York) filter. The filter was first washed with 3 × 10 mL ethanol and finally with 3 × 10 mL water. The filter was shaken vigorously with 2 mL pyrogen-free water, and endotoxin in the residue was measured using a kinetic turbidimetric LAL test as described in Ph.Eur. (Council of Europe, 2005). In brief, the method was based on the quantitative relationship between the endotoxin concentration and the time (onset time) needed for the reaction mixture to reach a predetermined absorbance. The LAL reagent (Cambrex Bioscience – BioWhittaker, MD, USA) had a sensitivity of 0.094 IU/mL and an *E. coli* 055:B5 endotoxin 250 pg/IU (Cambrex Bioscience – BioWhittaker, Walkersville, MD, USA) was used as standard. To validate the assay, the test solutions were spiked with endotoxin (0.25 IU/mL) and the recovery was estimated. Recovery in the range 50–200% was considered satisfactory. For use in cell culture, OVA was purified to remove endotoxins by using an EndoTrap® red kit (Profos, Regensburg, Germany), according to the operating procedures of the manufacturer. Purifying the OVA solution (10 mg/mL) reduced the endotoxin content from 25.6 IU/mg to 1.2 IU/mg, i.e. by more than 95%. The endotoxin concentration in DEHP was less than 2×10^{-4} IU/mg.

2.4. Generation of test atmospheres and aerosol characterization

DEHP was dissolved in acetone in concentrations ranging from 0.0043 to 9% w/v. The DEHP solutions were delivered from a glass syringe to a Pitt no. 1 aerosol generator (Wong and Alarie, 1982) by means of an infusion pump (New England Medical Instruments Inc., Medway, MA, USA). OVA was dissolved in 0.9% saline, and the feeding solutions containing 33 mg/L or 1% w/v OVA were aerosolized using a Pari Star nebulizer (PARI GmbH, Starnberg, Germany). The concentration of $\text{Al}(\text{OH})_3$ in the positive control group was 0.5% w/v, which in combination with 33 mg OVA/L gave rise to an air concentration of $67 \pm 15 \text{ mg/m}^3$. For simultaneous OVA and DEHP exposure, the aerosols were mixed and led to a glass/stainless-steel exposure chamber (Clausen et al., 2003). Aerosols were collected from the breathing zone of the mice. In the acute study, DEHP concentrations were determined gravimetrically (Clausen et al., 2003). In the experiment series with mixed DEHP and OVA exposures, OVA and the two highest exposure concentrations of DEHP were measured gravimetrically, whereas the two lower exposure concentrations of DEHP were determined by means of TD–GC–MS as described previously (Clausen et al., 2004). For the TD–GC–MS analyses, air from the exposure chamber was led through a Perkin–Elmer Tenax TA adsorption tube. The DEHP concentrations in the exposure chamber were 0.022 ± 0.0029 , 0.094 ± 0.0032 , 1.7 ± 0.64 and $13 \pm 3.2 \text{ mg/m}^3$, respectively, and the OVA concentrations were 0.14 ± 0.04 and $70 \pm 11 \text{ mg/m}^3$ for the low and high-dose solutions, respectively. In none of the exposure experiments did acetone exceed 1900 ppm, which is below the airway irritation threshold (Larsen et al., 2004). The OVA and OVA + $\text{Al}(\text{OH})_3$ aerosols had been characterized with respect to size distribution in previous studies (cf. Hansen et al., 2007a). Estimated depositions of DEHP aerosols in the respiratory and gastrointestinal tracts are given in Table 1.

2.5. Collection of respiratory parameters

The Notocord Hem (Notocord Systems SA, Croissy-sur-Seine, France) data acquisition software was used to collect respiratory parameters. For each experiment, up to 10 mice were placed in body plethysmographs in the exposure chamber and animals were exposed head-only (Vijayaraghavan et al., 1994). Amongst other things, the acquisition program calculates the respiratory frequency (breaths/min), time of inspiration (ms), time of expiration (ms), time from end of inspiration until the beginning of expiration, termed time of brake (TB, ms), time from end of expiration until beginning of the next inspiration, termed time of pause (TP, ms), tidal volume (VT, mL) and mid-expiratory flow rate (mL/s). Comprehensive descriptions of the breathing parameters have been made elsewhere (Alarie, 1998; Larsen et al., 2000; Nielsen et al., 1999; Vijayaraghavan et al., 1994). Data acquisition and

Table 1
Estimated deposition of di-(2-ethylhexyl)phthalate (DEHP) aerosols

Concentration of DEHP in feeding solution	Number of exposures ^a	Air concentration during exposure (mg/m^3) ^b	Cumulated doses of DEHP ($\mu\text{g/kg}$ body weight) ^c		
			Inhaled	Deposited in lungs	Deposited in upper respiratory tract
0.0043% w/v	22	0.022 (0.0029)	14	3	7
0.023% w/v	22	0.094 (0.0032)	54	11	28
0.45% w/v	22	1.7 (0.64)	1011	200	520
9.0% w/v	22	13 (3.2)	7515	1485	3870

^a Exposures were 20 min/day and were performed daily for 5 days/week for 2 weeks and then weekly for 12 weeks.

^b Values are mean with S.D. in parenthesis.

^c Calculations of doses were performed as described previously (Hansen et al., 2007a). Deposited doses were based on characterization of the particle size distribution in the aerosol generated from the 9% w/v DEHP feeding solution.

Table 2
Overview and brief description of experiments

Experiment (Exp)	Aim of experiment	Exposure design	DEHP concentrations (mg/m ³)	Assessment based on
Exp I	Acute airway irritation assessment	Single DEHP exposure (inhalation)	3.7–300	Breathing parameters evaluated over a 60-min period
Exp II	Acute airway inflammation assessment	Single DEHP exposure (inhalation)	300	Inflammation evaluated in BAL 0, 6, 16, 24 and 48 after end of exposure
Exp III	Assessment of adjuvant effect and allergic airway inflammation	Repeated exposure to DEHP and OVA over 14 weeks (inhalation)	0.022–13	Allergic inflammation based on BAL cell composition. Adjuvant effect based on serum antibody levels and cytokine profile in <i>ex vivo</i> lymphocyte cultures

For full information, confer Section 2.

calculations were performed as described previously (Larsen et al., 2004).

DEHP exposure was evaluated for acute airway irritation, airway inflammation and adjuvant effect as shown in Table 2.

2.6. Acute airway irritation and inflammation

The aim of the acute experiments was to achieve knowledge about acute airway irritation and inflammation caused by DEHP alone. To assess the airway irritation effects of DEHP, groups of mice ($n = 8$) were exposed for 60 min to concentrations of DEHP of 3.7, 18.4, 31.6 and 300 mg/m³, respectively. Prior to exposure, a 15-min baseline period was recorded for each mouse. To assess exposure-dependent effects, the respiratory parameters during exposure were compared to baseline levels, i.e. each mouse served as its own control.

DEHP-induced airway inflammation was assessed by exposing groups of mice ($n = 7$ per group) to 300 mg/m³ DEHP for 60 min. 0, 6, 16, 24 and 48 h after end of exposure, bronchoalveolar lavage (BAL) was performed according to the procedure described below.

2.7. Repeated exposure protocol for sensitization

Groups of 10 mice were exposed 20 min per day, 5 days/week for 2 weeks, and then 20 min weekly for 12 weeks. Treatments were OVA control, OVA + DEHP (referred to as DEHP) or OVA + Al(OH)₃ (referred to as Al(OH)₃). DEHP concentrations were 0.022, 0.094, 1.7 and 13 mg/m³ and the OVA concentration was 0.14 mg/m³. OVA and DEHP were administered simultaneously by mixing aerosols from the two different aerosol generators. The mice in the OVA and Al(OH)₃ groups were exposed to the same concentration of acetone (vehicle) as mice in the DEHP exposure groups. After the 14 weeks, mice were exposed 20 min/day for three consecutive days to a nebulized 1% OVA solution.

2.8. Bronchoalveolar lavage procedure

BAL was performed by flushing the lungs four times with 0.8 mL of saline (0.9%) using a tracheal cannula. The BAL fluids recovered were pooled and centrifuged (500 × g, 10 min, 4 °C). The supernatant was discharged and the pellet was resuspended in a 100 µL PBS buffer containing heparin (20 IE/mL) and serum albumin (0.003%). The total numbers of cells were determined using a haemocytometer. For differential counts, cytospin preparations were made (Cytospin[®] 2, StatSpin[®] Inc., Norwood, MA, USA) (1000 × g, 4 min, RT). Slides were stained with May-Grünwald/Giemsa and all slides were inspected by the same technician. Cells were identified by standard morphology and differentiated into neutrophils, eosinophils, epithelial cells, lymphocytes and macrophages. For each slide, 200 cells were counted and the percentage of each cell type was calculated.

2.9. Lymph node cell assay and measurements of cytokine production

On termination of the experiment, superficial cervical (SLN), deep cervical (DLN) and mediastinal (MLN) lymph nodes were excised from the animals. Nodes were disintegrated by using a 70 µm nylon cell strainer (BD Falcon, from BD Falcon Biosciences, Bedford, MA, USA) and the same kind of lymph nodes from different animals ($n = 2–3$) within the same exposure group were pooled and lymphocytes counted. In order to obtain a sufficient number of lymph cells, lymph nodes were further pooled. Cells were cultured in 24-well plates (Nunc, Denmark), 1.0 mL cell suspension per well (2.7×10^6 cells/mL), in RPMI 1640 medium (Cambrex, Belgium) supplemented with Nutridoma (1%), gentamycin (50 µg/mL), and monothioglycerol (13.5 µL/L). Cells were either unstimulated or stimulated with 1 mg/mL of LPS-purified OVA. After 5 days, supernatants were harvested and analyzed for cytokines.

Concentrations of IL-4, IL-5, IL-10, IL-12p70 and IFN γ were measured by the BDTM Cytometric Bead Array Flex Set and FCAP ArrayTM Software on a FACSCalibur flow cytometer (all from BD Biosciences) according to the manufacturer's instructions.

2.10. Antibody production

From the second weekly exposure and throughout the study, blood samples were collected from the tail vein every second week. A volume of approximately 100 μ L blood was taken and sera were assayed for the content of OVA-specific IgE and IgG1. At the end of the experiment, mice were anaesthetized and blood samples were collected by heart puncture. Sera were assayed for content of OVA-specific IgE, IgG1 and IgG2a. ELISAs were performed as described previously (Larsen et al., 2003).

2.11. Statistics

Antibody levels, numbers of inflammatory cells in BAL fluid and lymphocytes in lymph nodes in the four DEHP groups were compared to the OVA control group by the Kruskal–Wallis test. If a statistically significant effect was apparent, the individual DEHP groups were further compared to the OVA group by the Mann–Whitney's *U*-test. A *p*-value of less than 0.05 was considered statistically significant. Levels of antibodies in the OVA and Al(OH)₃ groups were compared pairwise by the Mann–Whitney's *U*-test. Furthermore, to evaluate whether the numbers of inflammatory cells in the BAL fluids followed a concentration–response relationship, numbers of eosinophils, neutrophils and lymphocytes on the OVA control group and the four DEHP groups were evaluated by means of Spearman's rank correlation coefficient. Calculations were performed using the Minitab Statistical Software, Release 14 Xtra (Minitab Inc., PA, USA).

3. Results

3.1. Acute airway irritation and airway inflammation induced by DEHP

As only few effects were observed from the acute DEHP exposures, data are not shown. In the 1-h acute exposure experiment, DEHP did not cause sensory irritation, i.e. upper respiratory tract irritation, as indicated from the normal TB values at all exposure levels. However, the highest DEHP concentration gradually reduced the VT over the 60 min exposure period, giving rise to a maximum decrease of about 35% in the 50th–60th min of the exposure period. The reduced VT was associated with a minor (approximately 15%) increase in the respiratory rate, i.e. DEHP induced rapid, shallow breathing. No increase was seen on the TP.

Macrophage numbers in BAL fluid over time gave rise to a bell-shaped curve, with a maximum cell number after 16 h. However, no statistically significant difference in macrophage numbers was seen over time when data were analysed using the Kruskal–Wallis test, suggesting that DEHP did not induce inflammation, even at a concentration of 300 mg DEHP/m³.

3.2. Generation and characterization of aerosols

By weight, 92% of the DEHP particles had an aerodynamic diameter of $\leq 3.5 \mu\text{m}$, 57% were below 1.55 μm , 22% were below 0.93 μm whereas 8% were below 0.52 μm . In consequence, the DEHP particles were able to reach all levels of the respiratory tract (Raabe et al., 1988). More than 50% of the inhaled OVA and OVA + Al(OH)₃ was estimated to be deposited in the respiratory tract, which in total amounted to approximately 1 μg OVA during the 14 weeks of low-dose OVA exposures (Hansen et al., 2007a).

3.3. Mouse body and liver weights after repeated DEHP inhalations

The body weights of the mice were monitored throughout the study. At the end of the study, the mean weights and S.D. were 21.7 ± 1.1 , 22.4 ± 1.2 , 23.3 ± 1.4 , 21.6 ± 1.1 , 22.2 ± 0.4 and 20.6 ± 1.0 g in the OVA, 0.022 mg/m³ DEHP, 0.094 mg/m³ DEHP, 1.7 mg/m³ DEHP, 13 mg/m³ DEHP and Al(OH)₃ groups, respectively. The mean $\pm$ S.D. values of the liver weights (% of body weight) were 4.1 ± 0.5 , 4.3 ± 0.3 , 4.3 ± 0.3 , 4.5 ± 1.4 , 4.2 ± 0.3 and 4.5 ± 0.2 in the respective groups. Neither body nor liver weights differed significantly between the OVA and the DEHP groups, suggesting that DEHP did not have systemic toxic effects and that *in vivo* conversion of DEHP to its metabolite, mono-2-ethylhexyl phthalate (MEHP), did not reach levels giving rise to systemic PPAR α activation.

3.4. Serum antibody responses

Compared to the OVA control group, a statistically significant increase in OVA-specific IgE level was seen in the Al(OH)₃ control group, whereas no effect was seen on the IgE response in any of the DEHP exposure groups (Figs. 1A and 2A). DEHP did, however, increase the level of OVA-specific IgG1 antibody in an exposure-dependent manner and the production was significantly increased in the 13 mg/m³ group compared to the OVA control group. Also, the control

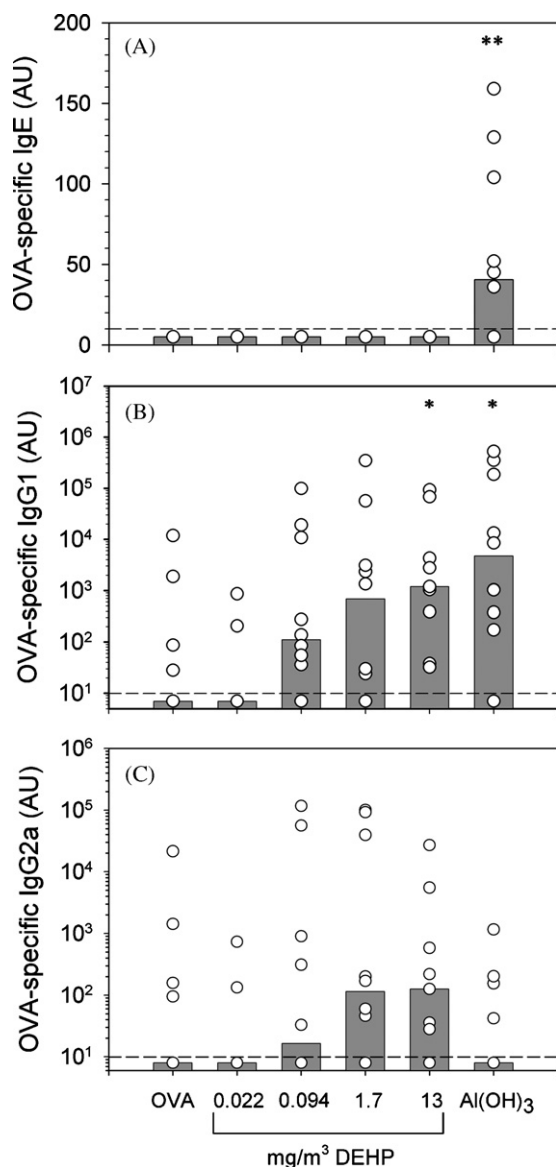


Fig. 1. Production of OVA-specific IgE, IgG1 and IgG2a antibodies. Groups of 9–10 mice were exposed to aerosols of OVA only or OVA together with 0.022, 0.094, 1.7, 13 mg/m³ DEHP or Al(OH)₃. Mice were exposed 5 days/week for 2 weeks, hereafter weekly for 12 weeks. Serum was collected after three final exposures to a 1% OVA solution. All exposures were for 20 min. The OVA-specific IgE (A), IgG1 (B) and IgG2a (C) production of the individual mice is given in arbitrary units (AU). Bars indicate the median of the group and the broken line shows the detection limit of the assays. Significant differences when compared to OVA group are indicated by * $p < 0.05$ or ** $p < 0.01$.

group exposed to Al(OH)₃ showed a significantly elevated IgG1 level (Figs 1B and 2B). No statistically significant adjuvant effect was seen on IgG2a in any of the groups, although IgG2a formation tended to increase with increasing DEHP exposure (Fig. 1C),

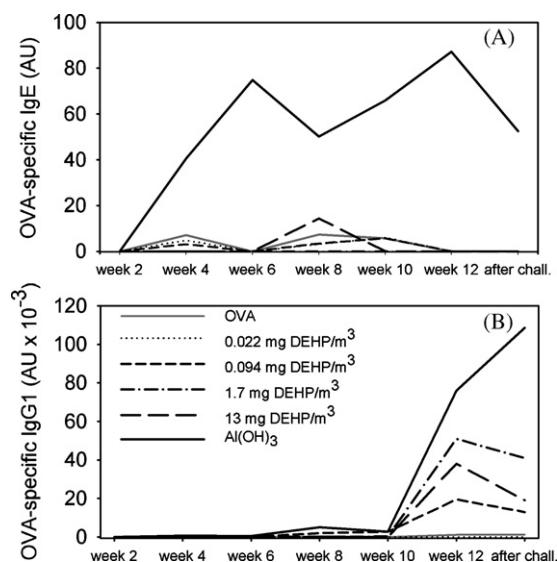


Fig. 2. Development of OVA-specific IgE and IgG1 antibodies over time. Groups of 9–10 mice were exposed to aerosols of OVA only or OVA together with 0.022, 0.094, 1.7, 13 mg/m³ DEHP or Al(OH)₃ as indicated in legend to Fig. 1. The mean OVA-specific IgE (A) and IgG1 (B) productions were analyzed after every second weekly exposure and after the last OVA exposure. Antibody production is given in arbitrary units (AU) as the mean value of the group.

but correlation analysis showed a non-significant association.

3.5. Inflammatory cells in BAL fluid

Numbers of eosinophils increased with increasing concentrations of DEHP, giving rise to a significantly elevated number after exposure to 13 mg DEHP/m³ compared to the OVA control group. The number of eosinophils was, however, much higher in the Al(OH)₃ control group (Fig. 3). The number of neutrophils also increased with increasing dose in the DEHP exposure group and was significantly higher than the OVA control group in the 13 mg DEHP/m³ group as well as in the Al(OH)₃ control group. Also, an increased number of lymphocytes was observed in the 13 mg DEHP/m³ group. No overall exposure-dependent effect was seen on the numbers of alveolar macrophages ($p = 0.2$, Kruskal–Wallis). However, when tested pair-wise with the OVA control group, both the 13 mg DEHP/m³ group ($p = 0.02$) as well as the Al(OH)₃ group ($p = 0.04$) had significantly reduced numbers of macrophages.

Correlation analysis revealed that the number of eosinophils and lymphocytes, but not neutrophils, followed concentration–response relationships.

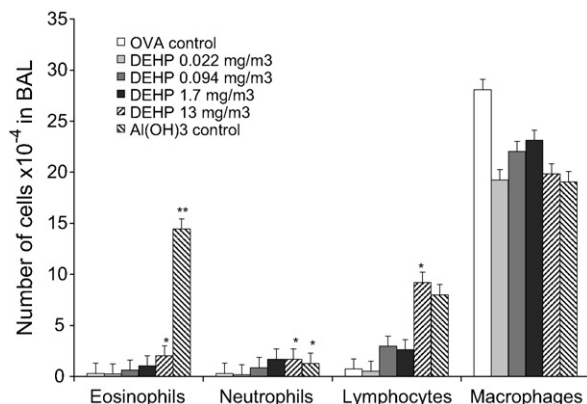


Fig. 3. Inflammatory cells in bronchoalveolar lavage fluid (BAL). Mice were exposed to aerosols of OVA only or OVA together with 0.022, 0.094, 1.7, 13 mg/m³ DEHP or Al(OH)₃ as indicated in legend to Fig. 1. Total and differential cell counts were performed following three final exposures to a 1% OVA solution. Mean and S.E.M. (error bar) from groups of 8–10 mice are presented. Significant differences when compared to the OVA control group are indicated by * $p < 0.05$ or ** $p < 0.01$.

3.6. *Ex vivo* cytokine production in draining lymph node cell cultures

The local lymph nodes play an important role in the development of an immune response (Lambrecht, 2005). To examine the role of the local lymph nodes after inhalational exposures, SLNs, DLNs, and MLNs were collected and pooled from 2–3 mice. Lymphocytes were counted. Compared to the OVA control group, the numbers of lymphocytes in the SLNs were nominally higher in the Al(OH)₃ and 13 mg/m³ DEHP groups, but only the 13 mg/m³ DEHP group reached statistical significance ($p = 0.03$, Mann–Whitney's *U*-test, data not shown). Al(OH)₃ decreased the number of lymphocytes in the MLNs ($p = 0.03$), whereas no effect of DEHP or Al(OH)₃ was seen on the DLNs.

To determine cytokine profiles of draining lymph nodes, single cell suspensions of lymph nodes were used for *ex vivo* cultures and stimulated for 5 days with medium or OVA (1 mg/mL). In general, cytokine production was more prominent in the culture with cells from the MLNs compared to SLN and DLN cultures. IL-4 secretion (Fig. 4A) in the OVA-stimulated cultures of MLNs showed that the highest level was seen in the Al(OH)₃ group. IL-4 secretion was very low in DLNs and SLNs.

With respect to IL-5 and IL-10 secretions, the highest levels in MLNs were seen in the Al(OH)₃ group followed by the 13 mg/m³ DEHP group. In general, MLN cells from the DEHP exposure groups had higher IL-5 and IL-10 secretion than the OVA control group

(Fig. 4B, C). The same trend was seen in DLNs and SLNs.

Secretion of INF γ was most prominent in the MLNs. Here, the lowest response was seen in the Al(OH)₃ control group, whereas the OVA and DEHP groups had higher INF γ levels. INF γ secretion was much lower and the pattern less clear in the DLNs and SLNs (Fig. 4D).

IL-12p70 was undetectable in all supernatants (data not shown).

4. Discussion

Based on the macrophage numbers in BAL fluid, the present study showed that no statistically significant airway inflammation occurred after a single 60 min exposure to 300 mg DEHP/m³.

This study also showed that DEHP did not induce acute airway irritation after exposure of up to 31.6 mg/m³. Only when mice were exposed to a very high concentration of DEHP (300 mg/m³), was rapid shallow breathing observed. This pattern has previously been observed in mice exposed to MEHP (Larsen et al., 2004).

Since the type of irritation induced by DEHP is comparable to that seen after MEHP exposure, it can be speculated that DEHP may be degraded in the lungs to MEHP by hydrolytic cleavage of one of the ester bonds. In the study assessing the acute airway effects of MEHP (Larsen et al., 2004), it was shown that MEHP exposure gave rise to reduced VT and elongation of the TP. A 35% reduction in VT was seen at a MEHP concentration of 4 mg/m³ (calculated from Larsen et al., 2004), whereas the no-observed-effect level (NOEL) for the breathing parameter TP was 5.2 mg/m³. As the 300 mg/m³ DEHP gave rise to a 35% reduction in VT, but no effect on TP, it is possible that the acute effect of DEHP on the airways is mediated through MEHP. Assuming that the entire effect of DEHP on the airways can be ascribed to newly formed MEHP, the accumulation of newly formed MEHP in the airways is 4/300, i.e. approximately 1%. The expected relatively slow degradation process of DEHP to MEHP might also explain the slow and gradual decrease in VT over the 60 min exposure period.

In conclusion, acute DEHP exposure failed to induce airway inflammation, while it induced airway irritation at only very high concentrations.

MEHP is a PPAR- α and - γ agonist (Hurst and Waxman, 2003). PPAR α activation causes liver enlargement in rodents (Lake et al., 1987), which may serve as an indicator of systemic PPAR α effects. PPAR α and - γ agonists may affect the immune system, showing both pro- and anti-inflammatory effects (Clark, 2002). The

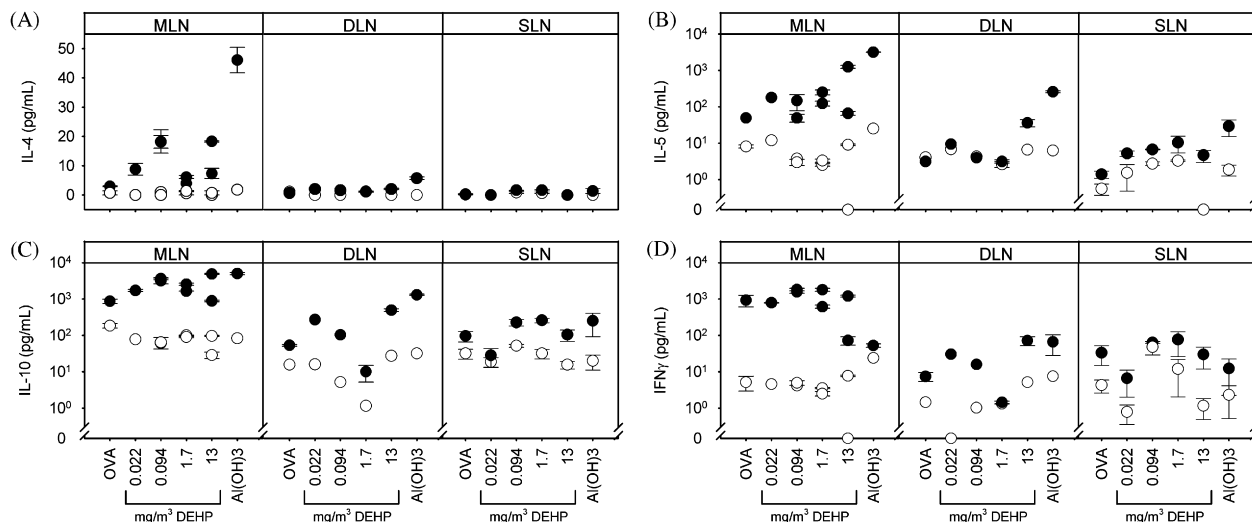


Fig. 4. Cytokine production in *ex vivo* cultures of draining lymph node cells. Groups of 9–10 mice were exposed repeatedly to aerosols of OVA only or OVA together with 0.022, 0.094, 1.7, 13 mg/m³ DEHP or Al(OH)₃ as described in legend to Fig. 1. After three exposures to a 1% OVA solution, superficial cervical (SLN), deep cervical (DLNs) and mediastinal (MLN) lymph nodes were collected. Cells were incubated for 5 days with medium (white circles) or OVA (black circles). IL-4 (A), IL-5 (B), IL-10 (C), and IFN γ (D) were measured in supernatants. Tests were performed in replicates if enough cells were available in the pooled groups. MLNs were pooled from 3–10 mice in each group whereas SLNs were pooled from 2 to 5 mice and results shown as mean $\pm$ S.E.M. of the replicates. DLNs were pooled from all mice in each group.

fact that liver weights were not increased in the present study indicates that the amount of absorbed MEHP, formed from DEHP, after repeated exposures of up to 13 mg/m³ was not sufficient to induce peroxisome proliferation. This observation is in line with a similar inhalation study (Hansen et al., 2007b), reporting that 0.4 mg/m³ MEHP did not induce peroxisome proliferation.

In the present study, Al(OH)₃ selectively enhanced a Th2-type immune response giving rise to high IgE and IgG1 levels, but low IgG2a production. The Th2 response induced by Al(OH)₃ is further supported by the cytokine production, as MLN cell cultures from the Al(OH)₃ group had high IL-5 and IL-10 secretion, but low INF- γ secretion after restimulation with OVA. The adjuvant effect of DEHP was seen on the IgG1 production, whereas no effect was seen on IgE. Although not significant, there was a tendency that DEHP increased the IgG2a level. This may suggest that DEHP stimulates a mixed Th1/Th2 response, although Th2-dominant. Also, the cytokine profile of DEHP suggests a mixed Th1/Th2 immune response, as both IFN- γ and IL-5 were high in lymph node cell cultures. Previous studies with BALB/c mice showed that subcutaneous administration of DEHP and OVA significantly elevated the IgG1 response, whereas no significant adjuvant effect was seen on the IgE and IgG2a levels (Larsen et al., 2001). The study also showed that Al(OH)₃ gave rise to significant adjuvant effects on both IgE and IgG1 levels.

Thus, the results in the present inhalation study using the same mouse strain and the same allergen (OVA) are in accordance with results obtained after subcutaneous administration. A mixed Th1/Th2 immune response of DEHP has also been observed after intraperitoneal injections of DEHP in combination with OVA into 129/Sv mice (Larsen and Nielsen, 2007). The 129/Sv strain responded to DEHP with elevated levels of IgG1 and IgG2a whereas no effect was seen on IgE. In contrast, Al(OH)₃ stimulated the IgE and IgG1 production, but had no effect on the IgG2a level.

Since DEHP stimulates production of IgG1 rather than IgE, the relevance of IgG1 should be addressed, when translated into the human system. In humans, the protective effect of immunotherapy is partly achieved by induction of IgG4 (Francis and Larche, 2005), which blocks the allergen–IgE interaction (Ejrnæs et al., 2006). The protective effect of IgG antibodies has also been observed in mice in studies of anaphylaxis (Strait et al., 2006; von Garnier et al., 2000) and in immunotherapy studies, where mouse IgG was able to block the binding of IgE from allergic patients to the allergen (Herz et al., 2004). In mice, decreased IgG1 and increased IgG2a have been associated with the development of mucosal tolerance towards inhaled allergens (McMenamin et al., 1994). Furthermore, IgG1 in mice is an anaphylactic antibody (Oshiba et al., 1996), but only in combinations with high allergen doses (Finkelman et al., 2005). IgG1 is co-regulated with IgE via the IL-4/Th2 path-

Table 3

Mean and maximum concentrations of DEHP in air measured in studies from different countries

Study	# Obs	Mean conc. ($\mu\text{g}/\text{m}^3$)	Max. conc. ($\mu\text{g}/\text{m}^3$)
Personal sampling PM _{2.5} + vapour (Adibi et al., 2003)			
Poland	30	0.43	1.1
USA	30	0.22	0.41
Denmark, stationary sampling in an office (Clausen and Wolkoff, 1997)	9	0.17	0.50
Denmark, stationary sampling in four offices, a day-care centre and a classroom (Clausen et al., 1999)	12	0.33	1.1
USA, stationary sampling in homes (Sheldon et al., 1992)			
Day	115	0.14	1.0
Night	113	0.10	0.33
USA, homes (Rudel et al., 2003)	101	0.11	1.0
Japan, homes (Otake et al., 2001)	6	0.08	0.23
Germany (Fromme et al., 2004), homes,	59	0.19	0.62
kindergarten	73	0.60	2.25
USA, homes + workplaces (Rudel et al., 2001)	4	0.06	0.11
Japan (Otake et al., 2004) homes	27	0.32	3.13

way (Purkerson and Isakson, 1992), which may explain the use of IgG1 to assess immunogenicity or adjuvant effects in several murine systems (Kimber et al., 2003; Robinson et al., 1996; Yanagisawa et al., 2006) and as a risk factor for developing an IgE response in humans. Overall, the extent to which the IgG1 response should be considered a protective response or a risk factor for development of IgE is not clear at present.

The mouse is the predominant species used to model allergic airway disease in man (Taube et al., 2004). Although mouse models do not fully mirror human asthma, man and mouse share a number of key mediators of asthma. Among these, the airway inflammation associated with allergic asthma is usually characterized by an accumulation of eosinophils, T-lymphocytes, mast cell and neutrophils in the airway walls, lung tissue and airway lumen (Taube et al., 2004). In the 13 mg DEHP/m³ group, significantly elevated numbers of eosinophils, neutrophils and lymphocytes were observed. However, eosinophilic lung infiltration in the 13 mg DEHP/m³ group was less pronounced than in the Al(OH)₃ group. Pulmonary alveolar macrophages (PAM) have been reported to inhibit T-cell proliferation induced by lung and airway dendritic cells (Holt et al., 1993). Thus, substances that suppress or eliminate PAMs may be regarded as immune response stimulators. Both the Al(OH)₃ control group as well as the 13 mg DEHP/m³ group had significantly reduced macrophage numbers in BAL fluid. It could be speculated that the reduced numbers of PAMs in these groups were linked to the observed adjuvant effect. Also, DEHP has been reported to reduce alveolar

macrophage activity and even to be toxic to these groups (Bally et al., 1980; Shertzer et al., 1985).

We have previously published a study of the adjuvant effects of MEHP, which had identical effects on the immune system as DEHP, but at lower exposure concentrations (Hansen et al., 2007b). It is tempting to speculate that the adjuvant effects of DEHP may be caused by generation of MEHP in the airways. The NOEL of effects on airway inflammation and OVA-specific IgE production was 0.02 mg MEHP/m³. The lowest-observed-effect level (LOEL) was 0.4 mg MEHP/m³. In the present study, the NOEL and LOEL were 1.7 and 13 mg DEHP/m³, respectively. Assuming that the adjuvant effect of DEHP can be ascribed to newly formed MEHP, the accumulation of newly formed MEHP in the airways is between $0.02/1.7 = 1\%$ and $0.4/13 = 3\%$. This is in concordance with the effects observed from acute DEHP and MEHP exposures.

No effect, neither on antibody production nor on the number of inflammatory cells, was seen at the exposure level 1.7 mg DEHP/m³ air. Although not directly comparable, a 20 min exposure per week to 1.7 mg DEHP/m³ air corresponds to a continuous exposure of 3.4 μg DEHP/m³ air. Table 3 lists almost all existing studies of DEHP in air in indoor environments or at personal exposure. A wide variety of sampling and analytical techniques have been used, but all the techniques sample both gas phase and particle phase. Weschler (2003) has estimated that the distribution of DEHP between the gas phase and particle phase in typical indoor environments is roughly 1:5. Since total suspended particles

have been sampled in some of the studies, and since particles $>10\ \mu\text{m}$ only to a very small degree are deposited in the lungs (cf. Phalen and Oldham, 2007), the reported concentration may overestimate the actual exposure of the lungs. Adibi et al. (2003) report a field study among 30 women in Krakow, Poland assessing DEHP exposure using personal air sampling. DEHP exposure from particles $\leq 2.5\ \mu\text{m}$ and from DEHP vapour gave rise to a mean value of $0.43\ \mu\text{g}/\text{m}^3$ (maximum value $1.1\ \mu\text{g}/\text{m}^3$). At rest, BALB/c mice have a ventilation rate per kg body mass of about 1730 mL/min (calculated from data in Nielsen et al. (1999)), whereas the corresponding value for humans is about 100 (Despopoulos and Silbernagl, 1986). Using these data, the margin-of-exposure is $3.4/0.43 \times 1730/100 = 137$, when using the mean DEHP exposure or 53, if using the maximum value of $1.1\ \mu\text{g}/\text{m}^3$.

Thus, in conclusion, the present study does not support that realistic DEHP exposure levels give rise to adjuvant effect or allergic lung inflammation.

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