

Butyl benzyl phthalate: effects on immune responses to ovalbumin in mice

Rebecca Jane Dearman,^{a*} Catherine Jean Betts,^b Lorna Beresford,^a Laura Bailey,^a Helen Theresa Caddick^b and Ian Kimber^a

ABSTRACT: During recent decades the prevalence of IgE-mediated (atopic) allergic diseases in Western Europe and the USA has been increasing dramatically. It has been suggested that one possible cause is the presence in the environment of chemicals that may act as adjuvants, enhancing immune and allergic responses. Certain commonly used phthalate plasticizers such as butyl benzyl phthalate (BBP) have been implicated in this way. In the current experiments, the impact of BBP, applied by a physiologically relevant exposure route, on the vigour of immune responses induced in BALB/c strain mice has been examined. Mice were immunized via subcutaneous injection with the reference allergen ovalbumin (OVA) and received concurrent topical treatment with doses of BBP that induced significant changes in liver weight. The generation of specific anti-OVA IgE and IgG1 antibodies was measured by passive cutaneous anaphylaxis and by enzyme-linked immunosorbent assays, respectively. Topical administration of BBP was without impact on anti-OVA IgE antibody responses, regardless of whether BBP was applied locally or distant to the site of OVA immunization. However, same-site treatment with high-dose BBP (100 mg) did result in a modest elevation in anti-OVA IgG1 antibody production, a subclass of antibody used as a surrogate marker of IgE responses. Taken together with human exposure data, these results suggest that the doses of phthalate encountered in the home environment are unlikely to be a major factor contributing to the increased incidence of asthma and allergy in the developed world. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: phthalates; adjuvant; allergenicity; IgG1; IgE

Introduction

During the last four or five decades there has been a substantial increase in the prevalence of atopic allergy, including atopic dermatitis, and asthma in Western Europe and the USA (Anderson *et al.*, 2004; O'Connell, 2004; Simpson and Hanifin, 2006). Independent studies conducted in the UK and in Switzerland indicate that in more recent years the trend towards an increasing incidence for both childhood and adult atopic disease may be flattening out (Anderson *et al.*, 2007; Braun-Fahrlander *et al.*, 2004). Although genetic susceptibility is an important risk factor in the development of allergy and asthma (with several genetic polymorphisms having been identified that are associated with atopic disease; Contopoulos-Ioannidis *et al.*, 2007), the observed changes in prevalence have been too swift to have resulted from modification of the gene pool (Geha, 2000). It is generally acknowledged that changes in lifestyle, diet and the environment, such as air pollution, indoor air quality, endotoxin, tobacco smoke or viral infections, are likely to have driven the observed increases in disease prevalence (Devereux and Seaton, 2005; Gilmour *et al.*, 2006; Holgate *et al.*, 2007; Obihara *et al.*, 2007; Strachan, 1989). However, it is also now recognized that genes associated with asthma and atopy have important interactions with these environmental factors through epigenetic mechanisms that influence their expression: 'gene by environment interaction' (Holgate *et al.*, 2007; Koppelman, 2006). Further evidence in support of the hypothesis that lifestyle and environmental factors in Western industrialized countries encourage the development of atopic allergy comes from the observation that migrants from less industrialized countries where there is a lower prevalence of allergy

and asthma display increased susceptibility following migration (Rottem *et al.*, 2005).

It has been suggested that one possible cause of this increase is the presence in the environment of chemicals or pollutants that may act as adjuvants, enhancing immune and allergic responses. Apparently consistent with this is the observation that the biggest effects have been seen among children and young adults living in inner cities in the USA (Eggleston *et al.*, 1999). Additionally, on the basis of recent human and animal laboratory-based studies, it has been reported that particulate toxic pollutants, (particularly diesel exhaust particles) can enhance allergic inflammation and induce the development of allergic immune responses, possibly through the production of oxidative stress in airway cells (Gilmour *et al.*, 2006; Peterson and Saxon, 1996). One group of organic chemicals that has also been implicated is the phthalates. These are used commonly in the manufacture of epoxy resins, plasticizers and adhesives, and global production of phthalates has increased during the last 50 years to approximately 3.5 million metric tons annually (Cadogan and Howick, 1996). It has been claimed from epidemiological studies that increased total levels of phthalates

* Correspondence to: R. J. Dearman, Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK. E-mail: rebecca.dearman@manchester.ac.uk

a Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK.

b Safety Assessment, AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK.

in household dust are found in homes of subjects with asthma and/or allergic rhinitis. Bornehag *et al.* (2004) conducted a nested case-control epidemiological study and reported an association between the concentration in house dust of some phthalates and allergic symptoms in children. In that study, butyl benzyl phthalate (BBP) was associated with rhinitis and eczema, whereas di(2-ethylhexyl) phthalate (DEHP) was associated with asthma, with BBP apparently more active, given a stronger disease association despite being present at considerably lower concentrations. However, only an association, rather than a causal link, between phthalate exposure and symptoms was demonstrated as measurements of phthalates were made after the development of symptoms.

Other, somewhat conflicting, data derive from experimental models in mice in which the ability of phthalates to modulate immune responses to reference proteins has been investigated. It is generally accepted that allergy and asthma result from a preferential activation of type 2 T cells [producing cytokines such as interleukin (IL)-4, IL-5 and IL-13] and the production of IgE antibody, the key biological mediator of the disease (Johansson, 2006; Sampson, 2004). Some investigators have reported that exposure to phthalates such as DEHP by nonphysiological routes (subcutaneous or intraperitoneal injection) resulted in enhanced antibody and/or cytokine responses stimulated by a reference protein (Larsen *et al.*, 2001a,b, 2002; Lee *et al.*, 2004). In contrast, we and others have demonstrated previously that topical exposure to DEHP, even under conditions where marked systemic effects were detected on liver weight, failed to cause adjuvant effects (Butala *et al.*, 2004; Dearman *et al.*, 2008).

Given the epidemiological evidence that BBP may have a greater potential to influence allergic responses than DEHP, we have now explored the impact of BBP on the vigour of immune responses induced in BALB/c strain mice. Immunization was via subcutaneous injection with suboptimal doses of ovalbumin (OVA), the major chicken egg allergen, which is a common cause of food allergy and can provoke occupational asthma in exposed workers (Bernstein *et al.*, 1987; Sicherer and Sampson, 2006). The generation of specific anti-OVA IgE and IgG1 antibody has been utilized as the readout for the stimulation of type 2 responses, IgG1 antibody being regulated similarly to IgE in mice and frequently used as a surrogate marker of IgE responses (Snapper *et al.*, 1988).

Materials and Methods

Mice

Young adult (8–12 weeks old) female BALB/c strain mice (Barriered Animal Breeding Unit, Alderley Park, Cheshire) were used throughout these studies. Food and water were available *ad libitum*. The diet used was Special Diet Services Rat and Mouse no. 1 Maintenance Diet comprising primarily cereal products (Special Diets Services, Witham, UK). The ambient temperature was maintained at $22 \pm 3^\circ\text{C}$ and relative humidity was $50 \pm 20\%$ with a 12 h light/dark cycle. All experiments were carried out under the provisions of the UK Animals (Scientific Procedures) Act, 1986.

Exposure Regimens

Subcutaneous Exposure to Allergen Alone. Groups of mice ($n = 5$ or 10) were shaved on the nape of the neck and received a subcutaneous injection of 100 μl of various concentrations of OVA

(Grade V, 98% pure; Sigma Chemical Co., St Louis, MO, USA; 0.5, 10 or 100 $\mu\text{g ml}^{-1}$) in phosphate buffered saline (PBS). Ten and 15 days after the initiation of treatment, animals received a booster subcutaneous injection at the same site of 100 μl of OVA (0.05, 1 or 10 $\mu\text{g ml}^{-1}$; one-tenth of the dose administered on day 0) in PBS.

Subcutaneous Exposure to Allergen and Alum. Positive control sera were generated as follows. Groups of mice ($n = 10$) were shaved on the nape of the neck and received a subcutaneous injection of 100 μl of 10 $\mu\text{g ml}^{-1}$ OVA formulated in PBS in the presence of 0.27 mg ml^{-1} alum. Ten and 15 days after the initiation of treatment, animals received a booster subcutaneous injection at the same site of 100 μl of 1 $\mu\text{g ml}^{-1}$ OVA in PBS in the presence of 0.27 mg ml^{-1} alum.

Topical Exposure. In the majority of experiments, neat (100%) BBP (Sigma) was applied topically. In one experiment, a dose-response was performed in which various concentrations of BBP (50–10%) were formulated in acetone:olive oil (4:1; AOO). Animals ($n = 10$) received 50 μl of neat BBP or of BBP diluted to various extents in AOO on both shaved flanks or on the nape of the neck for 15 daily exposures (five consecutive exposures followed by two rest days). Control mice received 50 μl of AOO alone according to the same dosing regimen. OVA was administered subcutaneously in the nape of the neck as described above. Concurrent positive control animals received subcutaneous injections of 100 μl of 100 $\mu\text{g ml}^{-1}$ of OVA formulated in PBS on days 0, 10 and 15.

Terminal Liver Weight Assessment

The systemic effects of BBP were monitored by assessment of terminal liver weights.

Serum Preparation

Mice were exsanguinated 21 days after the initiation of exposure. Individual and pooled (on a treatment group basis) serum samples were prepared and stored at -20°C until analysis.

Anti-OVA IgG1 ELISA

OVA-specific IgG1 antibodies were detected using enzyme-linked immunosorbent assay (ELISA). Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with 100 $\mu\text{g ml}^{-1}$ of protein in PBS by overnight incubation at 4°C . The plates were blocked by incubation for a further 30 min at 37°C with 2% bovine serum albumin (BSA; Sigma) in PBS. Doubling dilutions of mouse serum samples (derived from individual animals; starting dilution ranging from 1:10 to 1:640) diluted in 1% BSA in PBS were added to consecutive wells and incubated for 3 h at 4°C . There followed a further incubation for 2 h at 4°C with goat anti-mouse IgG1 alkaline phosphatase antibody (STAR81A; Serotec, Kidlington, UK) diluted 1 in 8000 in 1% BSA in PBS. Substrate (4-nitrophenyl phosphate disodium hexahydrate dissolved in 1 M diethanolamine buffer containing 0.5 mM MgCl; pH 9.8) was added to the plate. The reaction was stopped after 30 min by the addition of 2 M NaOH. Between each incubation stage, the plates were washed with PBS containing 0.05% Tween 20. Substrate conversion was measured as optical density (OD) at 405 nm using an automated reader (Flow Laboratories, Irvine, UK). Negative control serum derived

from naive (untreated) animals and positive control serum derived from animals immunized subcutaneously with OVA in the presence of alum were analyzed in every assay.

Calculation of Arbitrary Units

In each ELISA, a positive control serum sample (derived from mice immunized subcutaneously with OVA in the presence of 0.27 mg ml^{-1} alum) was utilized to generate a standard curve. A series of doubling dilutions, usually starting at 1:80 dilution, was performed in duplicate and a mean OD 405 nm value was calculated for each serum dilution. The log-linear portion of the positive control curve was plotted. Arbitrary units (AU) were assigned to the log-linear portion of the curve as follows: AU of 350 was assigned to the serum dilution of 1 in 640, AU of 175 was assigned to the serum dilution of 1 in 1280, AU of 87.5 was assigned to the serum dilution of 1 in 2560, and so on. Each test sample was run in duplicate for 13 serial doubling dilutions, and those serum dilutions at which the OD 405 nm readings were within the log-linear portion of the standard curve was utilized to derive the test sample titre in AU. For each serum sample three readings were used to derive the AU.

Passive Cutaneous Anaphylaxis Assay

The presence in mouse serum of IgE antibodies was detected by homologous passive cutaneous anaphylaxis (PCA) assay. Pooled serum samples diluted to various extents with PBS were injected ($30 \mu\text{l}$) into the dermis of the ears of naive recipient mice ($n = 4$). Concurrent negative control serum samples (derived from naive animals) diluted to an equivalent extent with PBS were incorporated into every PCA assay. Two days later, 0.25 mg of relevant protein, together with Evans Blue dye (1.25 mg), in 0.25 ml of PBS were injected intravenously. Thirty minutes following challenge, mice were killed and the diameter of cutaneous reactions measured. A positive (IgE) response was recorded if the challenge resulted in a $>3 \text{ mm}$ blue lesion in the skin in the majority of recipient animals, with antibody titre recorded as the highest dilution of serum resulting in a positive PCA reaction.

Statistical Analyses

A one-way analysis of variance (ANOVA) was used to determine whether group means were significantly different. This was followed by the Dunnett's multiple comparison test, in which test groups were all compared with the negative control group (OVA alone), or were all compared with the positive control group ($10 \mu\text{g}$ of OVA) using PRISM GraphPad Software (La Jolla, CA, USA). Groups differing at the level of $p < 0.05$ were considered significant.

Results

Sighting studies were performed to confirm that the maximum dose of BBP selected ($100\% \text{ w/v}$) was without significant local toxicity or irritant effects. Despite topical application of up to $100\% \text{ BBP}$ daily for five consecutive days, there was no discernable treatment-related redness or thickening of the skin. Subsequently, in two independent experiments, groups of BALB/c strain mice ($n = 10$) received a subcutaneous injection in the nape of the neck of $1 \mu\text{g}$ of OVA, a concentration that has been shown previously to be suboptimal for IgG1 antibody production, or of $10 \mu\text{g}$

of OVA, a dose that provokes maximal IgG1 antibody expression (Dearman *et al.*, 2008). Further groups received the suboptimal dose of OVA ($1 \mu\text{g}$) and topical application of $100\% \text{ BBP}$ to the same site (neck) or to a distant site (flanks). In each of the experiments conducted, this dose of BBP had a significant ($p < 0.05$) impact on liver weight whether applied to the same or to the distant site compared with treatment with OVA ($1 \mu\text{g}$) alone. (Mean and SE for experiment 1: $1 \mu\text{g}$ OVA, $1.058 \text{ g} \pm 0.027$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ same site, $1.215 \text{ g} \pm 0.026$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ distant site, $1.365 \text{ g} \pm 0.064$. Experiment 2: $1 \mu\text{g}$ OVA, $1.209 \text{ g} \pm 0.033$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ same site, $1.484 \text{ g} \pm 0.034$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ distant site, $1.477 \text{ g} \pm 0.045$.) As reported previously (Dearman *et al.*, 2008), in both experiments conducted, subcutaneous injection of $1 \mu\text{g}$ of OVA resulted in suboptimal anti-OVA IgG1 antibody expression, with significantly ($p < 0.05$ or $p < 0.01$) higher levels of IgG1 antibody detected following exposure to $10 \mu\text{g}$ OVA (Fig. 1). Concurrent treatment at a distant site (flanks) with $100\% \text{ BBP}$ failed to impact significantly on anti-OVA IgG1 antibody production. However, treatment at the OVA immunization site (nape of the neck) with the same dose of BBP consistently resulted in a significant ($p < 0.05$ or $p < 0.01$) increase in anti-OVA IgG1 antibody production (Fig. 1) compared with the lower dose ($1 \mu\text{g}$) of OVA. Such were still considerably lower than those recorded following treatment with high dose OVA ($10 \mu\text{g}$). In a third experiment, however, in which similar effects of same site treatment with $100\% \text{ BBP}$ on liver weight were noted, no increase in anti-OVA IgG1 antibody responses was observed ($1 \mu\text{g}$ OVA, $158 \text{ AU} \pm 30$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ same site, $101 \text{ AU} \pm 12$; $1 \mu\text{g}$ OVA $100\% \text{ BBP}$ distant site, $109 \text{ AU} \pm 24$). Both doses of OVA (1 and $10 \mu\text{g}$) were optimal for anti-OVA IgE expression, with IgE titres of 1 in 32 or 1 in 64 achieved for these groups in both experiments. Similar high-titre IgE antibody (1 in 64) was detected following concomitant treatment with BBP.

In subsequent experiments, mice received subcutaneous injections of doses of OVA ($0.05 \mu\text{g}$) that were suboptimal for anti-OVA IgE antibody production in order to examine the adjuvant potential of BBP on this read out. Exposure to this concentration of OVA resulted in IgE antibody titres of between 1 in 4 and 1 in 16, compared with titres of 1 in 64 and 1 in 128 achieved following treatment with $10 \mu\text{g}$ of allergen (Fig. 2). Concurrent treatment with BBP, either local or distant to the site of immunization with OVA, had no significant effect on IgE antibody production with titres of 1 in 4 or 1 in 8 recorded (Fig. 2). As observed previously, local or distant application of BBP had a profound effect on liver weight, confirming the systemic effects of BBP under these conditions of exposure. (Mean and SE for experiment 1: $0.05 \mu\text{g}$ OVA, $1.164 \text{ g} \pm 0.023$; $0.05 \mu\text{g}$ OVA $100\% \text{ BBP}$ same site, $1.330 \text{ g} \pm 0.032$; $0.05 \mu\text{g}$ OVA $100\% \text{ BBP}$ distant site, $1.454 \text{ g} \pm 0.041$. Experiment 2: $0.05 \mu\text{g}$ OVA, $1.188 \text{ g} \pm 0.037$; $0.05 \mu\text{g}$ OVA $100\% \text{ BBP}$ same site, $1.395 \text{ g} \pm 0.041$; $0.05 \mu\text{g}$ OVA $100\% \text{ BBP}$ distant site, $1.466 \text{ g} \pm 0.043$.) This dose of OVA ($0.05 \mu\text{g}$) was also suboptimal for anti-OVA IgG1 antibody responses, with a mean titre of 20 AU achieved compared with approximately 100 and 600 AU following treatment with 1 and $10 \mu\text{g}$ of OVA, respectively (Fig. 3). In these experiments it was therefore possible to also examine the potential adjuvant effects of BBP exposure on anti-OVA IgG1 antibody production, albeit under conditions where relatively low-titre IgG1 antibody was provoked (Fig. 3). Interestingly, neither local nor distant treatment with $100\% \text{ BBP}$ had a significant adjuvant effect on anti-OVA IgG1 antibody responses in the two independent experiments conducted, with

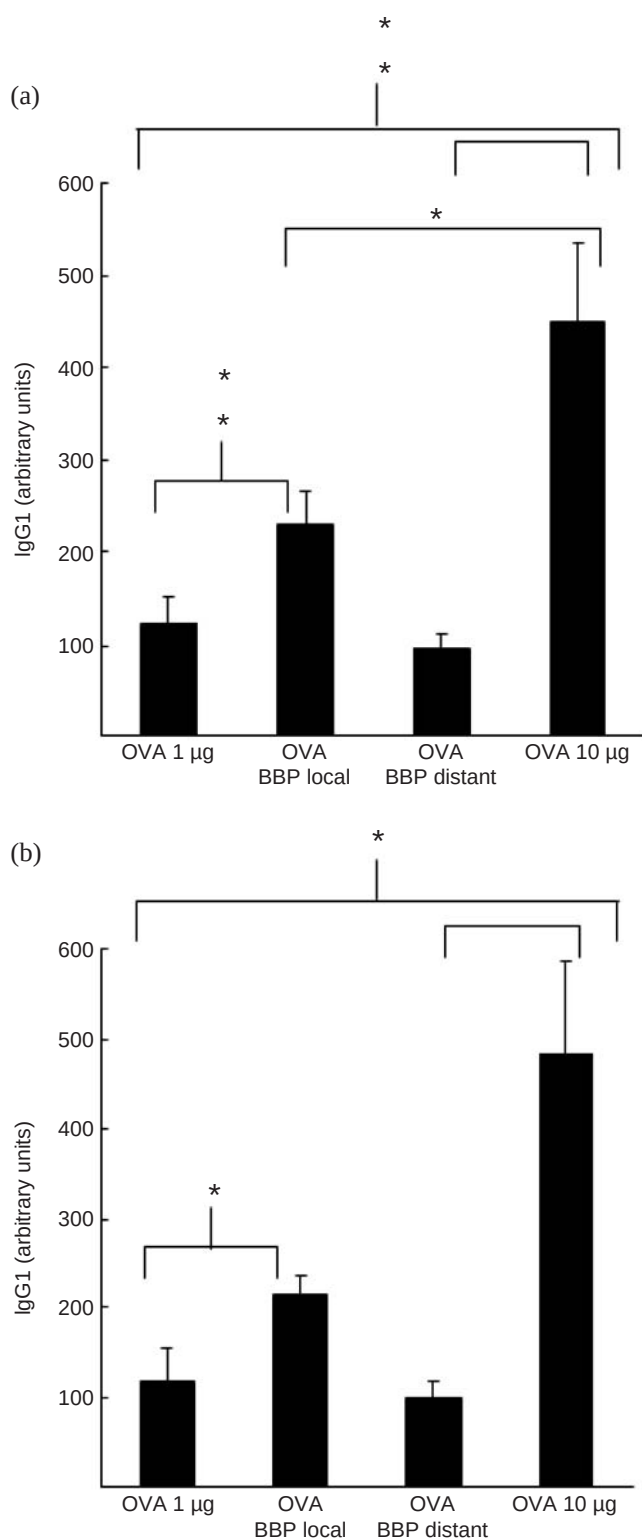


Figure 1. Influence of topically applied BBP on anti-OVA IgG1 antibody responses. BALB/c strain mice ($n = 10$; two independent experiments) were exposed to $1 \mu\text{g}$ of OVA in saline in the nape of the neck followed by booster doses of $0.1 \mu\text{g}$ in saline on days 10 and 15. OVA controls received no other treatment. In addition to subcutaneous administration of OVA, further groups of mice received $50 \mu\text{l}$ on both flanks or $100 \mu\text{l}$ on the nape of the neck of BBP (100%) on each of days 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17 and 18. Positive control mice received a primary subcutaneous immunization of $10 \mu\text{g}$ of OVA in saline followed by a booster dose of $10 \mu\text{g}$ in saline on days 10 and 15. Animals were killed 21 days after

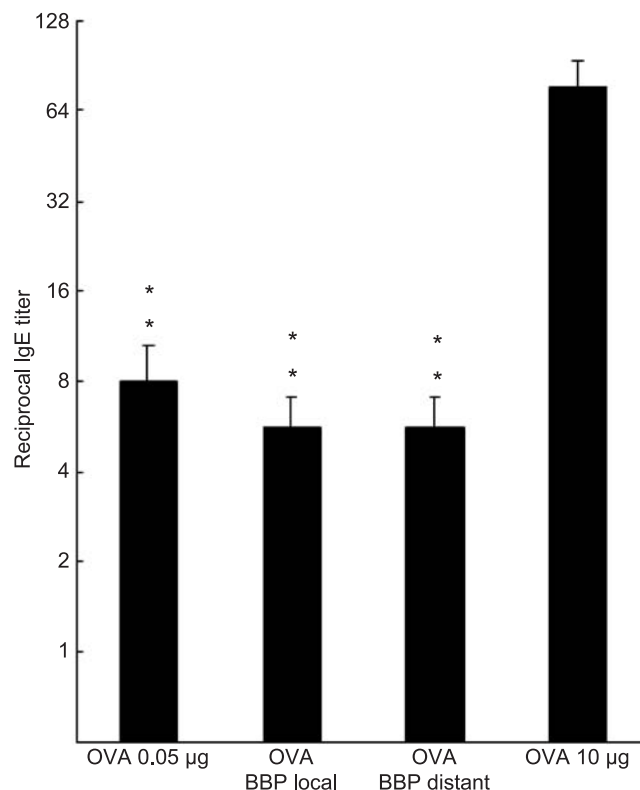


Figure 2. Influence of topically applied BBP on anti-OVA IgE antibody. BALB/c strain mice ($n = 20$ per group) were exposed to $0.05 \mu\text{g}$ of OVA in saline in the nape of the neck followed by booster doses of $0.005 \mu\text{g}$ in saline on days 10 and 15. OVA controls received no other treatment. In addition to subcutaneous administration of OVA, further groups of mice received $50 \mu\text{l}$ on both flanks or $100 \mu\text{l}$ on the nape of the neck of BBP (100%) on each of days 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17, and 18. Positive control mice received a primary subcutaneous immunization of $10 \mu\text{g}$ of OVA in saline followed by a booster dose of $10 \mu\text{g}$ in saline on days 10 and 15. Pooled serum samples were prepared ($n = 5$ per group) and anti-OVA IgE antibody content was measured by homologous PCA assay in four independent assays. IgE responses were determined as a function of the highest dilution of serum resulting in a positive PCA reaction. Data are displayed as mean and SE. Statistical significance of differences between groups was determined by Dunnett's multiple comparison test; $10 \mu\text{g}$ OVA vs $0.05 \mu\text{g}$ OVA, $0.05 \mu\text{g}$ OVA-distant BBP and $0.05 \mu\text{g}$ OVA-local BBP, $p < 0.01$ (**); $0.05 \mu\text{g}$ OVA vs $0.05 \mu\text{g}$ OVA-local BBP, and $0.05 \mu\text{g}$ OVA-distant BBP, not significant (NS).

very similar titres achieved to those recorded following exposure to $0.05 \mu\text{g}$ OVA alone (Fig. 3).

Finally, a dose-response experiment was conducted with BBP under conditions where significant adjuvant effects were observed

the initiation of exposure, serum samples were prepared and analyzed by ELISA for anti-OVA IgG1 antibody and responses determined as a function of AU. Data are displayed as mean and SE (a and b; two independent experiments). Statistical significance of differences in IgG1 antibody between groups was determined by Dunnett's multiple comparison test. Experiment 1: $10 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA and $1 \mu\text{g}$ OVA-distant BBP, $p < 0.01$ (**); $10 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-local BBP, $p < 0.05$ (*); $1 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-local BBP, $p < 0.01$ (**); $1 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-distant BBP, not significant (NS). Experiment 2: $10 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA and $1 \mu\text{g}$ OVA-distant BBP, $p < 0.05$ (*); $10 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-local BBP, NS; $1 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-local BBP, $p < 0.05$ (*); $1 \mu\text{g}$ OVA vs $1 \mu\text{g}$ OVA-distant BBP, not significant (NS).

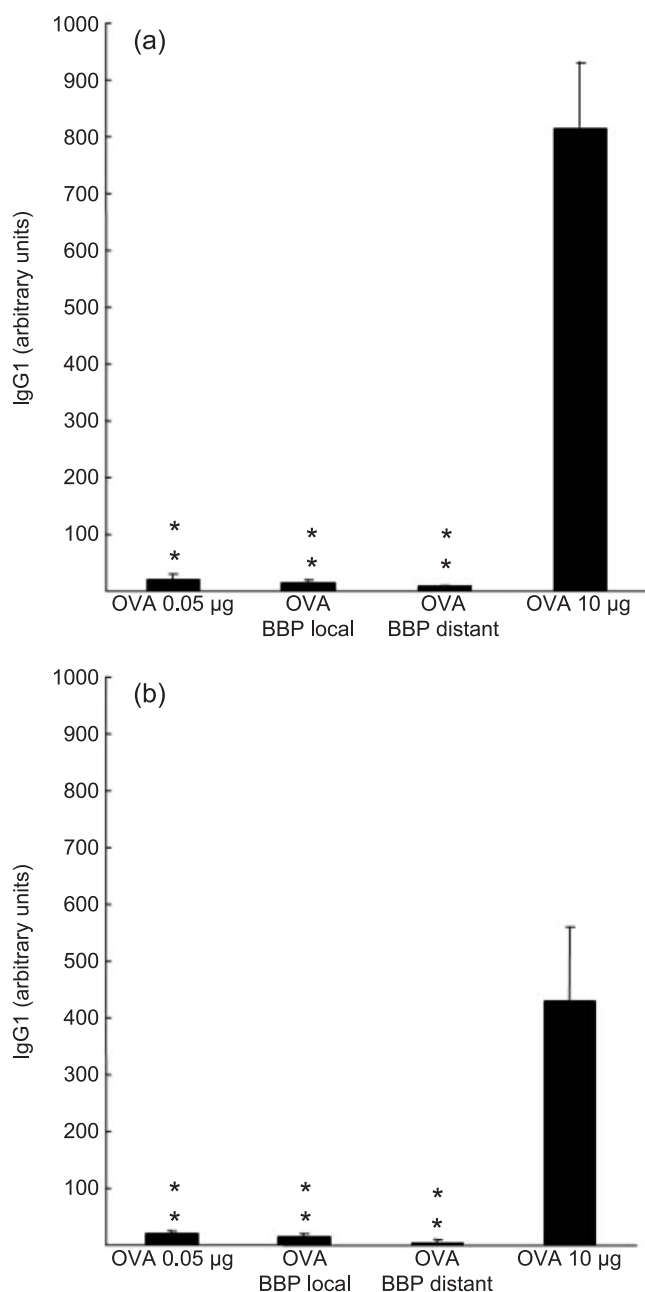


Figure 3. Influence of topically applied BBP on anti-OVA IgG1 antibody: impact on low titre responses. BALB/c strain mice ($n = 10$; two independent experiments) were exposed to $0.05 \mu\text{g}$ of OVA in saline in the nape of the neck followed by booster doses of $0.005 \mu\text{g}$ in saline on days 10 and 15. OVA controls received no other treatment. In addition to subcutaneous administration of OVA, further groups of mice received $50 \mu\text{l}$ on both flanks or $100 \mu\text{l}$ on the nape of the neck of BBP (100%) on each of days 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17 and 18. Positive control mice received a primary subcutaneous immunization of $10 \mu\text{g}$ of OVA in saline followed by a booster dose of $10 \mu\text{g}$ in saline on days 10 and 15. Serum samples were prepared 21 days after the initiation of exposure to OVA and analyzed by ELISA for anti-OVA IgG1 antibody and responses determined as a function of AU. Data are displayed as mean and SE (a and b; two independent experiments). Statistical significance of differences between groups has been determined by Dunnett's multiple comparison test; $10 \mu\text{g}$ OVA vs $0.05 \mu\text{g}$ OVA, $0.05 \mu\text{g}$ OVA–distant BBP and $0.05 \mu\text{g}$ OVA–local BBP, $p < 0.01$ (**); $0.05 \mu\text{g}$ OVA vs $0.05 \mu\text{g}$ OVA–distant BBP and $0.05 \mu\text{g}$ OVA vs $0.05 \mu\text{g}$ OVA–distant BBP, not significant (NS).

previously on IgG1 antibody responses (cf. Fig. 1); local (same site) exposure to BBP and $1 \mu\text{g}$ of OVA (Fig. 4). Mice ($n = 10$ per group) received subcutaneous injections of $1 \mu\text{g}$ of OVA in the nape of the neck and topical exposure to the same site of 100, 50, 10, 5 or 0% BBP–AOO. Positive control mice received subcutaneous injections of $10 \mu\text{g}$ of OVA in the nape of the neck. Compared with mice that received $1 \mu\text{g}$ of OVA–AOO, treatment with both 100 and 50% BBP resulted in a significant ($p < 0.01$) increase in liver weight, whereas lower doses were without effect [Fig. 4(a)]. All groups of mice treated with $1 \mu\text{g}$ OVA exhibited significantly ($p < 0.01$) lower levels of IgG1 than did the positive control ($10 \mu\text{g}$ OVA) group regardless of the presence of BBP. Although exposure to the highest dose of BBP (100%) resulted in somewhat higher levels of anti-OVA IgG1 antibody than those observed for the concurrent $1 \mu\text{g}$ OVA–AOO treatment group, such did not reach statistical significance in this experiment. The other doses of BBP were also without significant effect on anti-OVA IgG1 antibody production.

Discussion

The influence of topical exposure to BBP on anti-OVA antibody responses has been examined. Doses of OVA were utilized that resulted in sub-optimal IgE ($0.05 \mu\text{g}$) or IgG1 ($1 \mu\text{g}$) responses in order to provide conditions that would facilitate the detection of adjuvant effects on both antibody isotypes. In two out of four experiments a significant increase in anti-OVA IgG1 antibody production following subcutaneous immunization with $1 \mu\text{g}$ OVA and treatment at the same site with 100% BBP was observed. At lower doses of BBP (50%), even those for which a substantial elevation in liver weight was recorded, no effect on IgG1 antibody was recorded. Interestingly, there appeared to be a requirement for concurrent exposure to doses of OVA that provoked a relatively marked immune response. Thus, the relatively low levels of IgG1 antibody (10 – 20 AU) provoked by treatment with low dose OVA ($0.05 \mu\text{g}$) were unaffected by BBP whereas doses of OVA that stimulated IgG1 antibody levels of approximately 100 AU were enhanced by topical application of phthalate. Furthermore, there was no significant effect of BBP on anti-OVA IgE antibody responses, despite using concentrations that induced substantial increases in liver weight, confirming that there had been systemic exposure to the phthalate. It has been reported previously that the adjuvant effects of another phthalate, DEHP, were confined to the IgG1 antibody subclass without impact on IgE anti-OVA responses (Larsen *et al.*, 2001b, 2007). In these experiments the phthalate was administered subcutaneously and by inhalation, respectively (Larsen *et al.*, 2001b, 2007). As described previously, IgG1 antibody production in the mouse is often used as a surrogate for IgE antibody responses as IgG1 antibody is subject to similar cytokine regulation as is IgE antibody (Snapper *et al.*, 1988). However, it is IgE that is the primary anaphylactic antibody in humans and mice, particularly under conditions where there are low levels of antigen (Lehrer and Vaughan, 1976; Oshiba *et al.*, 1996). Murine IgG1 is considerably less effective than IgE at inducing mast cell degranulation, with a shorter receptor binding half life and considerably lower affinity (Lehrer and Vaughan, 1976; Ovary, 1982). Clearly, an observed adjuvant effect on murine IgG1 antibody in the absence of an effect on IgE antibody is of less concern with respect to potential human health effects than would be a marked impact on IgE.

It is also instructive to compare the results described herein with other publications describing the potential adjuvanticity of

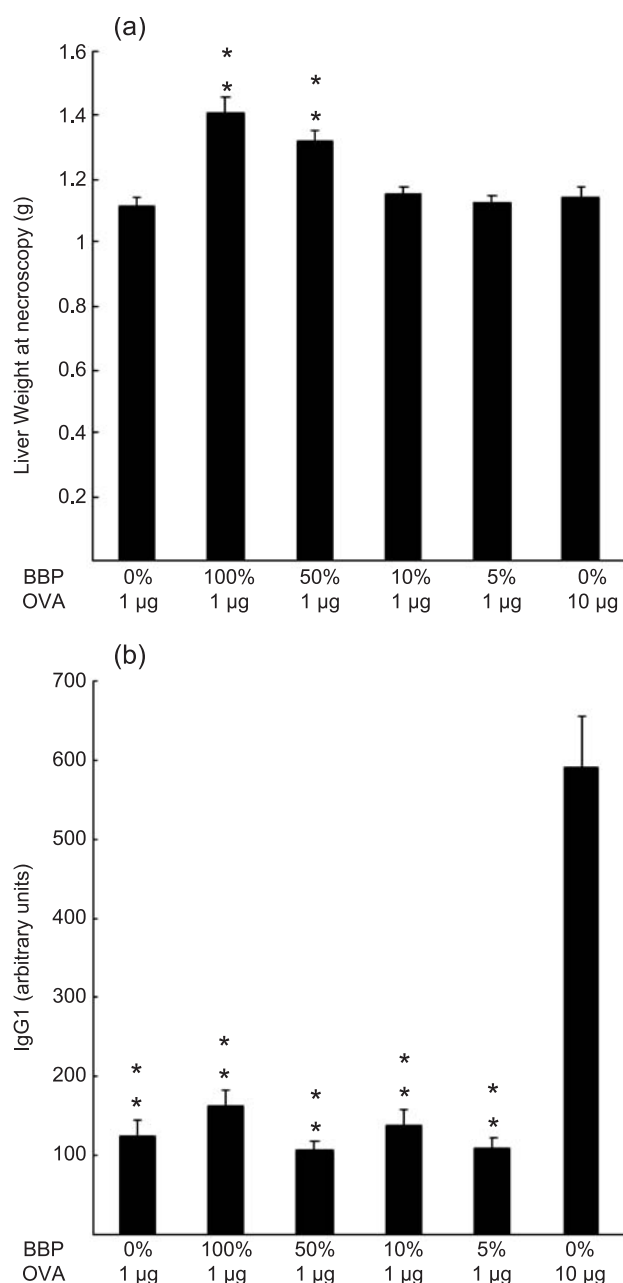


Figure 4. Influence of topically applied BBP on liver weight and anti-OVA IgG1 antibody: dose-response analyses. BALB/c strain mice ($n = 10$) were exposed to 1 µg of OVA in saline in the nape of the neck followed by booster doses of 0.1 µg in saline on days 10 and 15. In addition to subcutaneous administration of OVA, mice received 100 µl on the nape of the neck of various concentrations of BBP (100, 50, 10, 5 or 0%) on each of days 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17 and 18. Positive control mice received a primary subcutaneous immunization of 10 µg of OVA in saline followed by a booster dose of 10 µg in saline on days 10 and 15. Animals were killed 21 days after the initiation of exposure and total liver weights recorded on an individual animal basis. Results are displayed as mean and SE liver weight per group (a). Statistical significance of differences between groups has been determined by Dunnett's multiple comparison test; 1 µg OVA-AOO vs 1 µg OVA-100 or 50% BBP, $p < 0.01$ (**); 1 µg OVA-AOO vs 10 µg OVA or 1 µg OVA-10% or 5% BBP, not significant (NS). Serum samples were prepared and analyzed by ELISA for anti-OVA IgG1 antibody and responses determined as a function of AU. Data are displayed as mean and SE (b). Statistical significance of differences in IgG1 antibody between groups was determined by

BBP (Butala *et al.*, 2004; Larsen *et al.*, 2003). Butala *et al.* examined total serum IgE antibody production and expression of the type 2 cytokines IL-4 and IL-13 in cells derived from lymph nodes draining the site of topical application of 100% BBP in B6C3F1 strain mice. In contrast to the results described herein, high dose topical application of BBP failed to impact significantly on these parameters (Butala *et al.*, 2004). However, these measurements were made in the absence of immunization with a reference allergen or immunogen and thus did not address the issue of whether the phthalate had adjuvant effects on ongoing type 2 immune responses. These data suggest that an experimental system in which the effects of phthalates on antibody production in response to challenge are examined may be more sensitive to perturbation than is one that monitors effects in unchallenged animals. However, there are other differences including the mouse strain utilized (B6C3F1 vs BALB/c strain mice) and the fact that in the current studies the effects were observed for IgG1 antibody only, an antibody isotype that was not monitored by Butala *et al.* Larsen *et al.* (2003) also failed to observe adjuvant effects of BBP, despite using the same reference allergen (OVA) and very similar experimental read outs (specific IgG1 and IgE antibody production) to those described herein. In this case both the route of exposure and the dose of phthalate were very different (subcutaneous exposure to the phthalate and relatively low dose; 0.1 mg dose⁻¹ compared with 5 mg dose⁻¹), which presumably accounts for the differences, given the fact that only the highest dose (5 mg) was effective at enhancing antibody responses. The route of exposure to phthalates is also of some interest. Subcutaneous or intraperitoneal injection of some phthalates such as DEHP has been shown to result in adjuvant effects on antibody responses to reference proteins at comparatively low doses (Larsen *et al.*, 2001a,b, 2002; Lee *et al.*, 2004). Both routes are favoured for experimental immunization (van Zijverden and Granum, 2000) and the former also for vaccination; however neither are routes by which individuals would commonly be exposed to phthalates in the environment. Furthermore, it has been noted that it is possible to induce a 'false positive' adjuvant effect with subcutaneous injection as a result of local inflammation caused by injection trauma (Larsen *et al.*, 2003), consistent with the inflammatory properties of known adjuvants such as alum (Hamilton, 2003).

Recent studies have analyzed the potential routes of exposure to various phthalates using a scenario-based approach (Wormuth *et al.*, 2006). Phthalates are found in both air and dust of residential and occupational environments, are ubiquitous food contaminants and are found in many consumer goods and the important routes of exposure are oral, inhalation and dermal. Infants and toddlers receive the majority of their exposure to BBP and DEHP orally through the ingestion of both dust and food, whereas in adults the BBP burden derives primarily from inhalation of dust and food ingestion. Exposure to DEHP in adults is invariably through the diet (Wormuth *et al.*, 2006). Although the dermal route of exposure is apparently of little consequence in humans for these two phthalates, it must be noted that, following open topical application in experimental animals such as mice, the majority of the dose will be experienced orally as the animals will groom extensively. As an experimental system, the topical route of exposure is therefore more reflective of human exposure scenarios

Dunnett's multiple comparison test; 10 µg OVA vs 1 µg OVA-AOO or in the presence of 100, 50, 10 or 5% BBP, $p < 0.01$ (**); 1 µg OVA-AOO vs 1 µg OVA in the presence of 100, 50, 10 or 5% BBP, not significant (NS).

than is subcutaneous or intraperitoneal injection. Comparisons between likely levels of human exposure and the dose of BBP necessary to provoke adjuvant effects are also instructive. In the experiments described herein, adjuvant effects on IgG1 antibody only were observed at doses of approximately $2500 \mu\text{g kg}^{-1}$ body weight day⁻¹ of BBP. The calculated average intake of BBP in the most susceptible group (infants) is approximately $1 \mu\text{g kg}^{-1}$ body weight day⁻¹, suggesting that normal human exposure to BBP is unlikely to be at sufficient levels to cause adjuvant effects. Similar conclusions have been drawn by other authors when examining the effects of inhalation of the main metabolite of the phthalate DEHP, mono-2-ethylhexyl phthalate (MEHP) (Larsen *et al.*, 2007). Consistent with previous experience of exposure to DEHP via the subcutaneous route, MEHP enhanced anti-OVA IgG1 antibody responses but was without impact on IgE antibody. The extrapolated no-effect level of DEHP was 3 mg m^{-3} , which compares with estimated indoor air exposures of approximately $1 \mu\text{g m}^{-3}$, giving several orders of magnitude as a safety margin (Larsen *et al.*, 2007).

It is of some interest that in the current series of experiments adjuvant effects were observed only when exposure to the phthalate was at the same topical site as the site of application of the reference allergen OVA. As described previously, when applied topically to mice without occlusion as in the current investigations, the majority of the phthalate will be ingested due to grooming habits of the animals. The dose received orally will be identical whether the phthalate has been applied to the same or to a site distant to the site of administration of the allergen. Thus it is the fraction of the dose that is received through the skin that is the immunogenically relevant dose. This suggests that the mechanism of action of the phthalate is a perturbation of the local (cutaneous) immune system. Although there were no visible macroscopic signs of local inflammation even after topical application of 100% BBP, such may well result in the local production of cytokines and other inflammatory mediators. It has been reported that BBP and its metabolites activate murine and human peroxisome proliferator activated receptor (PPAR) alpha and PPARgamma (Hurst and Waxman, 2003). Recent reports have suggested that activation of PPARs can impact on the development of cutaneous immune responses, with PPAR activation inhibiting migration of Langerhans cells (the antigen presenting cells of the epidermis) and the subsequent development of contact hypersensitivity reactions (Angeli *et al.*, 2003). Furthermore, PPAR agonists have been reported to inhibit the production of IL-12, a cytokine that drives differentiation of T cells to the type 1 phenotype (Raikwar *et al.*, 2005; Jacobsen *et al.*, 2006) rather than the type 2 phenotype, which is permissive for IgE and IgG1 antibody production. This may provide for a mechanism for the local effects of topically applied BBP on the induction of immune responses to OVA in mice.

In the current experiments we have demonstrated that local topical treatment with high-dose BBP enhanced anti-OVA IgG1 antibody responses but was without effect on IgE antibody. Similar topical exposure to DEHP was without significant influence on immune responses (Butala *et al.*, 2004; Dearman *et al.*, 2008; Imai *et al.*, 2006). Other authors using less physiological routes of exposure (subcutaneous or intraperitoneal) have shown that certain phthalates have the potential to impact on the quality of immune response, causing changes consistent with activation of type 2 T cells. Taken together with human exposure data, these results suggest that doses of phthalate encountered in the home environment are unlikely to be a major factor contribut-

ing to the increased incidence of asthma and allergy in the developed world.

Acknowledgements

This work was funded in part by a grant from the phthalates working party of the European Chemical Plasticizers Industry (ECPI) and the American Chemical Council (ACC).

References

- Anderson HR, Ruggles R, Strachan DP, Austin BR, Burr M, Jeffs D, Standring P, Steriu A, Goulding R. 2004. Trends in the prevalence of symptoms of asthma, hay fever and eczema in 12–14 year olds in the British Isles, 1995–2002: questionnaire survey. *Br. Med. J.* **328**: 1052–1053. DOI: 10.1136/bmj.38057.583727.47.
- Anderson HR, Gupta R, Strachan DP, Limb ES. 2007. 50 years of asthma: UK trends from 1955 to 2004. *Thorax* **62**: 85–90. DOI: 10.1136/thx.2006.066407.
- Angeli V, Hammad H, Staels B, Capron M, Lambrecht BN, Trottein F. 2003. Peroxisome proliferator-activated receptor gamma inhibits the migration of dendritic cells: consequences for the immune response. *J. Immunol.* **170**: 5295–5301.
- Bernstein DI, Smith AB, Moller DR, Gallagher JS, Aw TC, London M, Kopp S, Carson G. 1987. Clinical and immunologic studies among egg-processing workers with occupational asthma. *J. Allergy Clin. Immunol.* **80**: 791–797.
- Bornehag CG, Sundell J, Weschler CJ, Sigsgaard T, Lundgren B, Hasselgren M, Hagerhed-Engman L. 2004. The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case–control study. *Environ. Health Perspect.* **112**: 1393–1397. DOI: 10.1289/ehp.7187.
- Braun-Fahrlander C, Gassner M, Grize L, Takken-Sahli K, Neu U, Stricker T, Varonier HS, Wüthrich B, Sennhauser FH. The Swiss Study on Childhood Allergy and Respiratory symptoms with respect to Air Pollution (SCARPOL) team. 2004. No further increase in asthma, hay fever and atopic sensitization in adolescents living in Switzerland. *Eur. Respir. J.* **23**: 407–413. DOI: 10.1183/09031936.04.00074004.
- Butala JH, David RA, Gans G, McKee RH, Guo TL, Peachee VL, White KL. 2004. Phthalate treatment does not influence levels of IgE or Th2 cytokines in B6C3F1 mice. *Toxicology* **201**: 77–85. DOI: 10.1016/j.tox.2004.04.004.
- Cadogan DF, Howick CJ. 1996. Plasticizers. In *Kirk–Othmer Encyclopedia of Chemical Technology*, 4th edn, Kirk–Othmer (ed.), Vol. 19. John Wiley and Sons: New York; 258–290.
- Contopoulos-Ioannidis DG, Kouri IN, Ioannidis JP. 2007. Genetic predisposition to asthma and atopy. *Respiration* **74**: 8–12. DOI: 10.1159/000096833.
- Dearman RJ, Beresford L, Bailey L, Caddick HT, Betts CJ, Kimber I. 2008. Di-(2-ethylhexyl) phthalate is without adjuvant effect in mice on ovalbumin. *Toxicology* **244**: 231–241. DOI: 10.1016/j.tox.2007.11.017.
- Devereux G, Seaton A. 2005. Diet as a risk factor for atopy and asthma. *J. Allergy Clin. Immunol.* **115**: 1109–1117. DOI: 10.1016/j.jaci.2004.12.1139.
- Eggleston PA, Buckley TJ, Breyse PN, Wills-Karp M, Kleeberger SR, Jaakkola JJ. 1999. The environment and asthma in U.S. inner cities. *Environ. Health Perspect.* **107**: 439–450.
- Geha RS. 2000. Allergy, a disease of the internal and external environments. *Current Opin. Immunol.* **12**: 615–617. DOI: 10.1016/S0952-7915(00)00152-7.
- Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA. 2006. How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. *Environ. Health Perspect.* **114**: 627–633. DOI: 10.1289/ehp.8380.
- Hamilton JA. 2003. Nondisposable materials, chronic inflammation, and adjuvant action. *J. Leukoc. Biol.* **73**: 702–712. DOI: 10.1189/jlb.0103037.
- Holgate ST, Davies DE, Powell RM, Howarth PH, Hiatchi HM, Holloway JW. 2007. Local genetic and environmental factors in asthma disease pathogenesis: chronicity and persistence mechanisms. *Eur. J. Respir.* **29**: 793–803.
- Hurst CH, Waxman DJ. 2003. Activation of PPARalpha and PPARgamma by environmental phthalate monoesters. *Toxicol. Sci.* **74**: 297–308. DOI: 10.1093/toxsci/kfg145.
- Imai Y, Kondo A, Iizuka H, Maruyama T, Kurohane K. 2006. Effects of

- phthalate esters on the sensitization phase of contact hypersensitivity induced by fluorescein isothiocyanate. *Clin. Exp. Allergy* **36**: 1462–1468. DOI: 10.1111/j.1365-2222.2006.02574.x.
- Jacobsen MA, Petersen RK, Kristiansen K, Lange M, Lillevang ST. 2006. Peroxisome proliferator-activated receptor alpha, delta, gamma1 and gamma2 expressions are present in human monocyte-derived dendritic cells and modulate dendritic cell maturation by addition of subtype-specific ligands. *Scand. J. Immunol.* **63**: 330–337. DOI: 10.1111/j.1365-3083.2006.01745.x.
- Johansson SG. 2006. The discovery of immunoglobulin E. *All. Asthma Proc.* **27**: S3–S6.
- Koppelman GH. 2006. Gene by environment interaction in asthma. *Curr. Allergy Asthma Rep.* **6**: 103–111.
- Larsen ST, Hansen JS, Thygesen P, Begtrup M, Poulsen OM, Nielsen GD. 2001a. Adjuvant and immunosuppressive effect of six monophthalates in a subcutaneous injection model with BALB/c strain mice. *Toxicology* **169**: 37–51. DOI: 10.1016/S0300-483X(01)00484-X.
- Larsen ST, Lund RM, Nielsen GD, Thygesen P, Poulsen OM. 2001b. Di-(2-ethyl) phthalate possesses an adjuvant effect in a subcutaneous injection model with BALB/c mice. *Toxicol. Lett.* **125**: 11–18.
- Larsen ST, Lund RM, Nielsen GD, Thygesen P, Poulsen OM. 2002. Adjuvant effect of di-*n*-butyl-, di-*n*-octyl-, di-iso-nonyl-, and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c strain mice. *Pharmac. Toxicol.* **91**: 264–272. DOI: 10.1034/j.1600-0773.2002.910508.x.
- Larsen ST, Lund RM, Thygesen P, Poulsen OM, Nielsen GD. 2003. Investigation of the adjuvant and immuno-suppressive effects of benzyl butyl phthalate, phthalic acid and benzyl alcohol in a murine injection model. *Food Chem. Toxicol.* **41**: 439–446. DOI: 10.1016/S0278-6915(02)00248X.
- Larsen ST, Hansen JS, Hansen EW, Clausen PA, Nielsen GD. 2007. Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl) phthalate and ovalbumin in BALB/c mice. *Toxicology* **235**: 119–129. DOI: 10.1016/j.tox.2007.03.010.
- Lee MH, Park J, Chung SW, Kang BY, Kim SH, Kim TS. 2004. Enhancement of interleukin 4 production in activated CD4 + T cells by diphthalate plasticizers via increased NF-AT binding activity. *Int. Arch. Allergy Immunol.* **134**: 213–222. DOI: 10.1159/000078768.
- Lehrer SL, Vaughan JH. 1976. Properties of mouse homocytotropic and heterocytotropic antibodies. *J. Allergy Clin. Immunol.* **57**: 422–429. DOI: 10.1016/0091-6749(76)90057-9.
- O'Connell EJ. 2004. The burden of atopy and asthma in children. *Allergy* **59**: 7–11.
- Obihara CC, Kimpen JL, Beyers N. 2007. The potential of mycobacterium to protect against allergy and asthma. *Curr. Allergy Asthma Rep.* **7**: 223–230.
- Oshiba A, Hammelman E, Takeda K, Bradley KL, Loader JE, Larsen GL, Gefand GW. 1996. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig)E and IgG1 in mice. *J. Clin. Invest.* **97**: 1398–1408. DOI: 10.1172/JCI118560.
- Ovary Z. 1982. Recent insight into class-specific properties of murine immunoglobulins obtained with the help of monoclonal antibodies. *Int. Arch. Allergy Appl. Immunol.* **69**: 385–392.
- Peterson B, Saxon A. 1996. Global increases in allergic respiratory disease: the possible role of diesel exhaust particles. *Ann. Allergy Asthma Immunol.* **77**: 263–270.
- Raikwar HP, Muthian G, Rajasingh J, Johnson C, Bright JJ. 2005. PPARgamma antagonists exacerbate neural antigen-specific Th1 response and experimental allergic encephalomyelitis. *J. Neuroimmunol.* **67**: 99–107. DOI: 10.1016/j.neuroimm.2005.06.026.
- Rottem M, Szyper-Kravitz M, Shoenfeld Y. 2005. Atopy and asthma in migrants. *Int. Arch. Allergy Immunol.* **136**: 198–204. DOI: 10.1159/000083894.
- Sampson HA. 2004. Update on food allergy. *J. Allergy Clin. Immunol.* **113**: 805–819. DOI: 10.1016/j.jaci.2004.03.014.
- Sicherer SH, Sampson HA. 2006. Food allergy. *J. Allergy Clin. Immunol.* **117**: S470–S475. DOI: 10.1016/j.jaci.2005.05.048.
- Simpson EL, Hanifin JM. 2006. Atopic dermatitis. *Med. Clin. North Am.* **90**: 149–167. DOI: 10.1016/j.mcna.2005.09.002.
- Snapper CM, Finkelman FD, Paul WE. 1988. Regulation of IgG1 and IgE production by interleukin 4. *Immunol. Rev.* **102**: 51–75.
- Strachan DP. 1989. Hay fever, hygiene, and household size. *Br. Med. J.* **299**: 1259–1260.
- Van Zijverden M, Granum B. 2000. Adjuvant activity of particulate pollutants in different mouse models. *Toxicology* **152**: 69–77. DOI: 10.1016/S0300-483X(00)00293-6.
- Wormuth M, Scheringer M, Vollenweider M, Hungerbühler K. 2006. What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? *Risk Anal.* **26**: 803–824. DOI: 10.1111/j.1539-6924.2006.00770.x.