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Review

An assessment of the ability of phthalates to influence immune and allergic responses

Ian Kimber*, Rebecca J. Dearman

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

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ABSTRACT

It has been suggested that one possible contributor to the increasing prevalence of atopic (IgE-mediated) allergic diseases and asthma in Europe and the US is exposure to chemicals that may act as adjuvants. Certain commonly used phthalate plasticisers, such as di-(2-ethylhexyl) phthalate, have been implicated in this regard. The evidence for the ability of phthalates to impact on immune and allergic responses has been examined, encompassing epidemiological investigations and results deriving from studies using experimental animals and from analyses *in vitro*. The epidemiological data provide some evidence that exposure to phthalates may be associated with increased risk of development of allergies and asthma, however, the lack of objective exposure information limits the interpretation. A variety of studies have been performed in mice to examine the influence of phthalate (delivered via various routes of exposure) to impact on immune responses. Measurement of antibody responses is the commonest read out, although other parameters of inflammation such as eosinophil infiltration and cytokine production have been used also. Although certain phthalates, when delivered at appropriate doses, and via an appropriate route, have been reported to impact on immune and inflammatory function in rodents, as yet no consistent pattern has emerged. Results ranged from potentiation of immune or inflammatory responses, to the absence of any effect, to inhibitory or immunosuppressive activity. In addition, comparatively low doses of phthalates have been associated with immune effects only when routes of administration (subcutaneous or intraperitoneal) are used that do not reflect, and are much less relevant for, opportunities for human contact with phthalates. There is clearly a case to be made for the design of more definitive animal studies that will allow development of a more detailed understanding of whether and to what extent, and under what conditions, phthalates are able to effect meaningful changes in immune function that may in turn impact on human health.

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Abbreviations: BBP, butyl benzyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DEP, diethylphthalate; DIBP, diisobutyl phthalate; DIDP, diisodecyl phthalate; DIHP, di-iso-hexyl phthalate; DINP, diisononyl phthalate; DOTP, bis-(2-ethylhexyl) terephthalate; DNBP, di-*n*-butyl phthalate; DNOP, di-*n*-octyl phthalate; DPHP, di(2-propylheptyl)phthalate; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; ip, intraperitoneal; KLH, keyhole limpet haemocyanin; LNC, lymph node cell; MBP, mono-*n*-butyl phthalate; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MIDP, monoiso-decyl phthalate; MINP, mono-iso-nonyl phthalate; MNBP, mono-*n*-butyl phthalate; MNC, mononuclear cell; MNOP, mono-*n*-octyl phthalate; OVA, ovalbumin; PVC, polyvinyl chloride; Th, T helper; TOTM, trioctyl trimellitate.

* Corresponding author. Tel.: +44 161 2751587; fax: +44 161 2755586.

E-mail address: ian.kimber@manchester.ac.uk (I. Kimber).

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1. Introduction to phthalates: chemistry, industrial use and exposure

Phthalates (or phthalate esters) are dialkyl or alkyl aryl esters of phthalic acid. Phthalates were first produced in the 1920s, with large-scale commercial production being introduced during the 1950s when polyvinyl chloride (PVC) (which is softened by phthalate esters) was first developed.

Phthalates are used in a wide variety of products and applications, and are found in gelling agents, medical devices, cosmetics, adhesives, lubricants, dispersants and emulsifying agents. They find use also in many household and consumer goods including, among others, PVC interior surface coverings, shower curtains, food wrappings, nail polish and plastic goods and kitchen plasticware. In the context of these applications among the most widely used phthalate have been di(2-ethylhexyl) phthalate (DEHP), and also butyl benzyl phthalate (BBP), diisobutyl phthalate (DIBP) and diethylphthalate (DEP). Of these DEHP was probably the most commonly used, but more recently longer chain phthalates such as diisodecyl phthalate (DIDP), diisononyl phthalate (DINP), or di(2-propylheptyl)phthalate (DPHP) are increasingly being used in place of DEHP (Wittassek et al., 2007) (Table 1).

Exposure to phthalates is widespread. Analytical surveys of metabolites of phthalate esters in urine, and in other body fluids, have confirmed the ubiquitous nature of exposure to multiple phthalates (Barr et al., 2003; Becker et al., 2004; Fromme et al., 2004; Wormuth et al., 2006; Hogeberg et al., 2008; Huang et al., 2009; Bornehag and Nanberg, 2009). Diet is the most important source of human exposure to phthalates, but inhalation and dermal exposures are also relevant. The highest levels of exposure (for individuals) derive from the use of DEHP in medical plastics and thus the intravenous route is also of some importance for those patients undergoing renal dialysis or blood transfusions, for example (Chou and Wright, 2006; Hansen, 2006). Previously, infant exposure may have derived from the use of childcare products and plastic toys. However, since 1999, the use of three phthalate esters (DEHP, DBP and BBP) in such articles has been banned completely in the European Union and three others (DNIP, DIDP and di-*n*-octyl phthalate [DNOP]) are banned from use in the same applications where the articles may be put into the mouth of a child 1999/815/EC; now included in Annex XVII, 51/52 of Regulation (EC) no 1907/2006). Since 2008, a similar restriction has been

in place in the US (Consumer Product Safety Improvement Act, 2008).

1.1. The biology and toxicology of phthalates

Against a background of widespread use and ubiquitous exposure there has been interest, and concern, about the ability of phthalates to cause adverse health effects in humans (Tickner et al., 2001; Hendorf et al., 2007; Swan, 2008; Kamrin, 2009; Lyche et al., 2009; Halden, 2010).

One important observation was that long term high level exposure to certain phthalates can cause non-genotoxic hepatocarcinogenesis in rodents, although following substantial mechanistic study over many years, it is now accepted widely that such effects are species-specific and not relevant for human health (Huber et al., 1996; International Agency for Research on Cancer Monographs, 2000; European Union Risk Assessment Report, 2008). Most recent research has focused on the effects of certain phthalate esters on reproduction, commonly on male reproductive development in experimental animals; the implication being that phthalates may be associated with abnormal sexual development and birth defects in humans (Lovenkamp-Swan and Davis, 2003; McKee et al., 2004; Salazar-Martinez et al., 2004; Swan et al., 2005; Latini et al., 2006; Hallmark et al., 2007; Sharpe, 2008; Howdeshell et al., 2008). However, the subject of this review article is on current interest in the possibility that phthalates may represent risk factors for the development of allergies and asthma (Bornehag et al., 2004; Mendell, 2007; Hansen et al., 2007; Jaakkola and Knight, 2008; Bornehag and Nanberg, 2009). The implication is that certain phthalates may act as adjuvants and thereby predispose to, and/or otherwise enhance, acquisition of allergic sensitisation. It is on this aspect of the biology of phthalates that the remainder of this article will focus.

2. Allergy and allergic sensitisation

Before considering whether, and to what extent, phthalate esters have the ability to impact on allergy, it is necessary to reflect upon the nature of allergic sensitisation, and the potential of adjuvants to influence that process.

The term allergy describes the adverse health effects that might result from the stimulation of a specific immune response. Allergic disease can take a wide variety of forms, can affect many organ

Table 1

List of di- and mono-phthalates displaying common names, abbreviations, structural formulae and CAS number where assigned.

Name	Acronym	Structural formula	CAS no.
Diethyl phthalate	DEP	$C_6H_4(COOC_2H_5)_2$	84-66-2
Di- <i>n</i> -butyl phthalate	DNBP	$C_6H_4[COO(CH_2)_3CH_3]_2$	84-74-2
Diisobutyl phthalate	DIBP	$C_6H_4[COOCH_2CH(CH_3)_2]_2$	84-69-5
Butyl benzyl phthalate	BBP	$CH_3(CH_2)_3OOC C_6H_4COOCH_2C_6H_5$	85-68-7
Di(2-ethylhexyl) phthalate	DEHP	$C_6H_4[COOCH_2CH(C_2H_5)(CH_2)_3CH_3]_2$	117-81-7
Diisononyl phthalate	DINP	$C_6H_4[COO(CH_2)_6CH(CH_3)_2]_2$	28553-12-0
Diisodecyl phthalate	DIDP	$C_6H_4[COO(CH_2)_7CH(CH_3)_2]_2$	26761-40-0
Diisohexyl phthalate	DIHP	$C_6H_4[COO(CH_2)_3CH(CH_3)_2]_2$	146-50-9
Di- <i>n</i> -octyl phthalate	DNOP	$C_6H_4[COO(CH_2)_7CH_3]_2$	117-84-0
Mono(2-ethylhexyl) phthalate	MEHP	$C_6H_4COOCH_2CH(C_2H_5)(CH_2)_3CH_3$	4376-20-9
Mono- <i>n</i> -octyl phthalate	MNOP	$C_6H_4COO(CH_2)_7CH_3$	117-81-7
Mono-isononyl phthalate	MINP	$C_6H_4COO(CH_2)_6CH(CH_3)_2$	No CAS number
Mono-isodecyl phthalate	MIDP	$C_6H_4COO(CH_2)_7CH(CH_3)_2$	No CAS number
Mono- <i>n</i> -butyl phthalate	MNBP	$C_6H_4COO(CH_2)_3CH_3$	131-70-4

systems and can be local, widespread or systemic. By definition allergy is dependent upon the provocation of an immune response, but the quality of that immune response may vary. For the purposes of this article the type of allergy that we will consider is atopic allergy – or allergy mediated by IgE antibody.

In common with other forms of allergic disease, IgE-mediated allergy develops in two phases; the first of which (induction phase) is associated with immunological priming and the acquisition of sensitisation. The second (or elicitation) phase is triggered when a sensitised subject encounters again the antigen (allergen) to which they are sensitised. Such encounter elicits an accelerated and more aggressive secondary immune response that results in local or more widespread inflammation and the clinical symptoms of allergy. In the context of IgE-mediated allergy, sensitisation is characterised by the development of an allergen-specific IgE antibody response. This IgE antibody associates with mast cells via specific membrane receptors, and at this point sensitisation has been acquired. Upon second or subsequent exposure to the inducing allergen a sequence of events is initiated resulting in allergic inflammation. The allergen cross-links IgE antibody on the surface of mast cells. This in turn causes mast cell activation and the release of preformed and newly synthesised mediators (including vasoactive amines and leukotrienes) that act in concert to provoke an inflammatory reaction. Such reactions may be local or systemic, and vary in intensity and severity from mild local erythema and oedema, to dramatic and catastrophic systemic anaphylaxis. Symptoms may affect one or more organs and may be manifested as atopic eczema in the skin, allergic rhinitis and asthma in the respiratory tract, allergic conjunctivitis in the eye, and by a variety of symptoms in the gastrointestinal tract. Allergy of this kind may be induced by a wide variety of antigens/allergens, including: food proteins, pollen proteins, wasp and bee stings, certain drugs and chemicals, inhaled moulds and dusts and animal dander (Kay, 2000).

The term atopy denotes a predisposition to mount IgE antibody responses and an increased susceptibility to developing allergic sensitisation. Atopy is in large part heritable. However, it has become clear that environmental factors and conditions may play important roles also in determining susceptibility. One manifestation of this is the fact that, in Western Europe and the US, there has been a very significant increase in the prevalence of atopic allergy and asthma since the second world war (Burney et al., 1990; Anderson et al., 2004; Latvala et al., 2005). The important point is that this increase in prevalence has been far too rapid to have been driven by alterations in the gene pool; the implication being that changes in environmental/domestic conditions, diet and/or life style have in some way impacted on susceptibility to this form of allergy (Strachan, 1989, 1996; Ring, 1997; Kimber, 1998; Holgate, 1998, 2004; Peden, 2000; Platts-Mills et al., 2005, 2006; von Mutius, 2007). The exact nature of factors that may influence susceptibility to atopic allergy have been keenly debated, and among the various candidates the ‘hygiene hypothesis’ has attracted both attention and controversy (Strachan, 1989, 1996; von Mutius et al., 1992, 1994; von Mutius, 2007; Ring, 1997; Yazdanbakhsh et al., 2002; Platts-Mills et al., 2005). This hypothesis argues that the increased prevalence of allergy and asthma is driven primarily by changes in the environments in which children are raised. A case is made that in ‘westernised’ societies, children are now better (and possibly over) protected from microbial challenge, and that this in turn causes or perpetuates a bias in the immune system such that children are predisposed to mount the quality of immune response that favours IgE antibody production and allergic sensitisation (Howarth, 1998; Prescott et al., 1998; Erb, 1999; Peden, 2000; Folkerts et al., 2000; Yazdanbakhsh et al., 2002; Umetsu et al., 2002). Although the hygiene hypothesis is attractive, and has commanded some support, there is a growing view that it may not provide the whole answer, and that other factors may also

be important. Among these are changes in diet, prenatal exposure to tobacco smoke, alterations in indoor air quality, and outdoor atmospheric pollution (and in particular exposure to diesel exhaust particles) (Peterson and Saxon, 1996; Salvi et al., 1999; Lux et al., 2000; Peden, 2000; Chen et al., 2004; Devereux and Seaton, 2005). In addition, there have been suggestions that exposure to chemical products or pollutants, in general, or to certain chemicals or classes of chemicals specifically, may encourage or enhance IgE antibody responses.

Thus, for instance, cleaning products used in domestic environments have been associated with an increased risk of asthma (Zock et al., 2001; Karjalainen et al., 2002; Medina-Ramon et al., 2003, 2005; Rumchev et al., 2004). Persuasive as these investigations are of an association between household cleaning and clinical disease, they do not necessarily implicate cleaning materials as being responsible for an increase in the incidence of allergic sensitisation. So, although the available data do suggest that some household cleaning materials are able to elicit or exacerbate respiratory reactions, there is no reason to suppose that those same materials are responsible for the increased prevalence of atopic allergy.

In 2005, a workshop was convened specifically to address whether and to what extent chemicals have played a role in the increased burden of atopic allergy in Europe and the US. A review based on the deliberations of participants at the workshop was published recently (Evans et al., 2008). In that article one conclusion drawn was that “*despite a popular belief that the increased burden of allergic diseases (and in particular asthma) that has been observed in westernised societies can be attributed (at least in part) to exposure to specific chemicals and/or to pollution in general, the available evidence suggests that a more complex relationship exists in which environmental and life style factors play important roles in this trend.*” Thus, based on the data reviewed at that time there was little reason to suppose that exposure to chemicals *per se* had increased susceptibility to allergic sensitisation.

It is against that background, that we now consider the human and experimental evidence available regarding the proposed ability of phthalate esters to induce or enhance the acquisition of allergic sensitisation.

Although there are occasional, but infrequent, case reports of contact urticaria syndrome to phthalates (Sugiura et al., 2002), the available evidence in humans clearly demonstrates that they lack significant potential to cause skin sensitisation and allergic contact dermatitis (Medeiros et al., 1999; Geier et al., 2004). Moreover, phthalates are uniformly negative in assays where chemical respiratory allergens test positive, including the guinea pig maximisation test and the murine local lymph node assay (Kimber et al., 2007). These data are consistent with the fact that as a class phthalate esters have little or no ability to form stable associations with proteins; reactivity with proteins being a prerequisite for the stimulation of allergic sensitisation by a chemical.

Although it is clear that they lack intrinsic sensitising potential, what has been suggested is that certain phthalates may act as adjuvants. It is appropriate, therefore, in advance of reviewing published data on the associations between phthalates and allergy, to consider briefly the nature and function of adjuvants.

3. Biology of adjuvants

The term adjuvant derives from the Latin *adjuvare*, meaning to assist or help. In an immunological context an adjuvant is a material that is able to enhance immune responsiveness without itself necessarily providing any specific antigenic stimulus. More formally an adjuvant can be defined as any substance that can accelerate, prolong or enhance a specific immune response, and in this context adjuvants find clinical application in augmenting responses to vaccines. Substances known to have adjuvant properties are

varied in nature, including aluminium salts (alum), oil-based materials (such as Freund's adjuvant and Freund's complete adjuvant), Cholera toxin and unmethylated CpG dinucleotide-containing DNA (Schijns, 2000; Klinman, 2003; Lindblad, 2004). The mechanisms through which adjuvants exert their influence on immune responses are complex, but as a general rule it is believed that adjuvant activity will necessarily embrace one or more of the following effects: enhanced delivery and/or deposition of antigen, stimulating the release of 'danger' signals (that promote the initiation of adaptive immune responses), and engaging the innate immune system, particularly through the activation of toll-like receptors (Schijns, 2000).

One important point in the context of allergic sensitisation is that some adjuvants may have qualitative, as well as quantitative, effects on specific immune responses. There is interest, in particular, in the possibility that some adjuvants (alum, Cholera toxin and Pertussis toxin sometimes being cited as candidates) favour preferentially the development of T helper (Th) 2-type immune responses and IgE antibody production (Freytag and Clements, 2005; De Gregorio et al., 2008; Kool et al., 2008). Whether or not some adjuvants are truly selective for Th2 responses, it is important to recognise that such qualitative effects will invariably be manifest against a background of a generalised increase in overall immunogenicity.

In considering whether phthalate esters are able in some circumstances to display adjuvant-like activity there is one other relevant issue. As indicated above, a commonly accepted definition of an adjuvant is of a material that is able to impact on the vigour (and/or quality) of a specific immune response without itself provoking an immune response. As alluded to above there is no evidence that phthalates have the potential to directly cause allergic sensitisation, nor is there any reason to suppose that they are intrinsically immunogenic. Taken together, the implication is therefore that any effect of exposure to phthalates on immune or allergic responses would be attributable to adjuvant-like properties, rather than via inherent immunogenicity.

4. Phthalates and immune function

4.1. Epidemiological studies

Occupational exposure to high concentrations of phthalate fumes, such as those inhaled by individuals performing hot-wire cutting of PVC film for example, has been linked to asthma and other respiratory symptoms (Polakoff et al., 1975; Andrasch et al., 1976; Falk and Portnoy, 1976; Brooks and Vandervort, 1977; Eisen et al., 1985; Markowitz, 1989; Nielsen et al., 1989). A variety of respiratory symptoms such as cough, work-related shortness of breath, wheezing and rhinitis, as well as a decline in FEV₁ were found to be increased in exposed workers compared with non-exposed reference groups. It should be noted however, that the majority of these studies were not adjusted for confounders. The role of exposure of adults at home or at work to PVC containing materials has been investigated also (Norback et al., 2000; Tuomainen et al., 2004; Jaakkola et al., 2006). A study of Swedish hospital workers revealed a higher risk of asthma symptoms in those individuals that occupied buildings showing signs of dampness-related degradation of DEHP in PVC flooring (Norback et al., 2000). In an intervention study it was shown that removal of damaged PVC flooring, and a consequential reduction of indoor pollutants such as 2-ethyl-hexanol, was associated with a significant reduction in the prevalence of respiratory, nasal and conjunctival symptoms (Tuomainen et al., 2004). In a case controlled population-based study a correlation was found between the presence of plastic wall coverings at work and an increased risk of asthma (Jaakkola et al., 2006). There has also been a number of epidemiological studies in which the association

of exposure to phthalates and the exacerbation of respiratory symptoms, such as bronchial obstruction or wheeze, or the development of atopic diseases (rhinitis or eczema), in children has been investigated (Oie et al., 1999; Jaakkola et al., 1999, 2000, 2004; Bornehag et al., 2004; Kolarik et al., 2008). In some of these studies, the relationship between PVC floorings or wall material and the development of asthma, allergy or related respiratory outcomes were measured (Oie et al., 1999; Jaakkola et al., 1999, 2000, 2004). In general, following adjustment for various confounders, risks of the various respiratory outcomes measured were reported to be increased in the presence of the plastic materials in the home environment. Two independent studies were conducted in Sweden (Bornehag et al., 2004) and in Bulgaria (Kolarik et al., 2008) in which the association between the phthalate content of house dust and allergic symptoms in children has been investigated. In both studies there was reported a statistically significant association between the concentration in house dust of DEHP and allergic symptoms (asthma or wheezing) in children. Despite the presence of higher levels of BBP in the Bulgarian homes compared with the Swedish homes, it was only in the Swedish study that an association with BBP in house dust and allergic symptoms (eczema and rhinitis) was found (Bornehag et al., 2004; Kolarik et al., 2008). It should be noted that both studies demonstrated an association, rather than a causal link, between DEHP exposure and symptoms, and, importantly, measurements of phthalate concentrations were made after the development of symptoms. That is, the degree of correlation between the levels of these materials during the relevant period of disease development and the period after which a diagnosis was made (when the measurements were performed) is unknown.

One important issue is that in most epidemiological studies it is not possible to exclude the possibility that products other than phthalates, that are also released from PVC materials following pyrolysis or dampness-related degradation, may contribute to the apparent associations between phthalate concentrations and allergic symptoms. Indeed, a recent systematic review of the role of exposure to phthalates and the risk of development of allergies and asthma concluded that although there was some evidence to suggest that phthalate exposure may be associated with increased risk, the lack of objective exposure information limited the interpretation of these epidemiologic data (Jaakkola and Knight, 2008). A slightly earlier review (Mendell, 2007) also noted that although there were strong associations between indoor residential chemical emissions (such as formaldehyde and phthalates) and respiratory health or allergy, causality was not demonstrated and that these risk factors may only be correlative indicators for truly causal exposures.

Relevant also in this context is a wider review of indoor chemicals and the development of airway allergic disease in which it was concluded that "there is little evidence that the indoor chemicals evaluated possess important adjuvant effects" (Nielsen et al., 2007). That same article also drew attention to a discordance between the apparent potential of certain phthalates in some experimental systems to display adjuvant-like properties, and the failure of epidemiological studies to disclose any evidence for phthalates acting as adjuvants in exposed humans (Nielsen et al., 2007).

4.2. Experimental studies

4.2.1. Effects of phthalates on antibody expression in vivo

The putative ability of phthalates to act as adjuvants for the development of immune responses to known protein allergens has been investigated in animal (almost exclusively mouse) models. In considering the conduct and interpretation of such models it is important to appreciate that the immunological response of greatest moment in the development of allergic sensitisation and of atopic allergic disease (and therefore the event of greatest rele-

vance when considering the possible contribution of the putative adjuvant activity of phthalates in driving susceptibility to allergy) is the production of specific IgE antibody. This is the primary effect molecule of atopic allergy. During the acquisition of sensitisation IgE antibody distributes systemically and associates, via specialised plasma membrane receptors, with mast cells. As described above, subsequent encounter with the inducing allergen causes mast cell activation resulting in inflammation and the signs and symptoms of an allergic reaction. Although specific IgE antibody is therefore the most relevant marker of allergic sensitisation, it is produced in very small amounts and for this (and other reasons) is difficult to measure accurately. As a consequence, other antibody read-outs are commonly used in addition to, or as surrogates for, specific IgE. Those most commonly used in mouse models are total plasma levels of IgE immunoglobulin (as opposed to specific IgE antibody), and the induction of IgG1 antibody responses. Total plasma levels of IgE are frequently elevated during immune responses to allergens, and IgG1 antibody production is regulated by mechanisms similar to those that control IgE responses (Snapper et al., 1988). It is of some importance for the interpretation of data derived from murine models that IgG1 is not only considerably less effective at stimulating mast cell degranulation than is IgE (Ovary, 1982), but also IgG1 antibody levels do not always mirror exactly the same pattern as IgE antibody responses. Thus, we have observed very different dose response profiles for IgG1 anti-ovalbumin (OVA) antibody responses compared with anti-OVA IgE antibody responses following subcutaneous administration of DEHP to mice (Dearman et al., 2008). A lack of correlation between IgG1 and IgE specific antibody production was recorded also for detergent enzymes and food proteins (Sarlot et al., 2005).

Another issue is the dose metric to be used for comparison between different types of exposures. Although dose/unit body weight is accepted generally for comparisons between species, and for routes of exposure such as oral or intraperitoneal (ip), it is unclear how appropriate this metric is for consideration, for example, of topical exposure. For the current comparisons we have therefore elected to use total dose per application as the dose metric for comparison, given that all experiments have utilised the same experimental animal (the mouse).

Experimental approaches have included examination of the ability of various phthalate monoesters and diesters (administered by a variety of routes of exposure) to influence specific antibody responses to a reference protein allergen, OVA, or to a reference immunogen (keyhole limpet haemocyanin; KLH) (Larsen et al., 2001a,b, 2002, 2003, 2007; Lee et al., 2004; Larsen and Nielsen, 2007, 2008; Dearman et al., 2008, 2009, 2010; Hansen et al., 2007). The impact of phthalates on the total serum concentration of IgE has also been investigated (Butala et al., 2004; Lee et al., 2004; Dearman et al., 2010). In many, but not all, instances the strain of mouse that is most susceptible to the development of IgE-mediated reactions, the BALB/c strain mouse, has been utilised.

The ability of phthalates to display adjuvant properties under these experimental conditions appears to be influenced very markedly by the route through which exposure to the phthalate is effected. Subcutaneous exposure to some phthalates appears to be particularly effective at causing augmentation of antibody responses to OVA, with relatively low doses of phthalate (0.1–40 µg phthalate per dose) impacting on the vigour of IgG1 and/or IgE antibody responses. When the adjuvant effects of various phthalate monoesters (the primary hydrolytic metabolites of phthalate diesters) were studied, for some chemicals (mono-2-ethylhexyl phthalate [MEHP], mono-*n*-octyl phthalate [MNOP] and mono-iso-nonyl phthalate [MINP]), both inhibitory “immunosuppressive” effects (statistically significant decreases in antibody production) and adjuvant effects (statistically significant increases in antibody production) were observed at different doses within the same

experiment (Larsen et al., 2001a). Thus, for example, subcutaneous treatment of mice with MINP at 20 µg inhibited anti-OVA IgE antibody production; whereas 2 µg of the same chemical enhanced IgE antibody responses. Moreover, in the same experiment 0.2 µg was found to inhibit anti-OVA IgG1 antibody production. Only inhibitory effects on IgE and IgG1 antibody levels were recorded following exposure to 2 µg mono-iso-decyl phthalate (MIDP), with administration of either 20 µg or 0.2 µg of phthalate being without impact on antibody production.

Using an identical experimental system, and similar concentration ranges, subcutaneous injection of mice with monobenzyl phthalate (MBZP), or mono-*n*-butyl phthalate (MBP), was without effect on either IgG1 or IgE antibody responses (Larsen et al., 2001a). The same investigators have also examined the immunomodulatory effects of various diester phthalates (Larsen et al., 2001b, 2002, 2003). Inhibitory activity was not seen with any of these compounds. However, treatment with 40 µg DEHP was reported to cause increased expression of IgG1 anti-OVA antibody, although it was without effect on IgE anti-OVA antibody levels (Larsen et al., 2001b). Other diester phthalates such as DNOP, DINP and DIDP augmented both IgG1 and IgE anti-OVA antibody responses at certain doses (Larsen et al., 2002). In the same experimental system, subcutaneous injection of phthalates that are structurally similar to DEHP, including BBP, bis-(2-ethylhexyl) terephthalate (DOTP) and triethyl trimellitate (TOTM), was found not to influence either IgG1 or IgE antibody responses (Larsen et al., 2003; Larsen and Nielsen, 2008). The authors speculated that the length of the carbon side chains is important in dictating the biological activity of phthalates in this respect; the suggestion being that a total of 16 carbon atoms distributed between the two ester groups confers maximum adjuvant activity (Larsen and Nielsen, 2008).

Intraperitoneal route of administration of phthalates has also been reported to have adjuvant effects at relatively low doses. Thus, in 129/Sv strain mice ip injection of DEHP (100 µg) with the reference allergen OVA resulted in a significant increase in IgG1 anti-OVA antibody, but was without effect on IgE antibody responses (Larsen and Nielsen, 2008). These data are comparable with previous observations that subcutaneous injection of DEHP with OVA in BALB/c strain mice enhanced specific IgG1 antibody without impacting on specific IgE responses (Larsen et al., 2001b).

Using a somewhat different protocol, other investigators have shown that ip administration of either DEHP or DINP (40–125 µg) to BALB/c strain mice enhanced the total serum concentration of IgE, and also increased levels of specific IgE antibody provoked by subcutaneous injection of the immunogen KLH (Lee et al., 2004). Diisononyl phthalate proved more effective than did DEHP for the augmentation of IgE anti-KLH antibody responses. In our own laboratory, and using a similar protocol, we also found that ip injection of DINP, but not of DEHP, was associated with an increase in IgG1 anti-KLH antibody production (Dearman et al., 2010).

In contrast, the general experience has been that exposure of mice to phthalates via routes that more closely reflect conditions of human contact (that is either oral or topical exposure), is without impact on antibody responses, or is considerably less effective, at mediating adjuvant-like effects. Oral exposure of BALB/c strain mice to 50 mg per dose DEHP or DINP was without influence on the total serum concentration of IgE, or on levels of IgG1 anti-KLH antibody induced by concurrent subcutaneous administration of the antigen (Dearman et al., 2010). The doses of phthalates used in those experiments, and that were without effect on antibody production or IgE levels, did, however, display significant systemic effects, measured as a function of increases in liver weight (Dearman et al., 2010). Topical exposure of B6C3F1 mice to high doses (up to 100 mg) of DEHP, BBP, DINP or di-iso-hexyl phthalate (DIHP) also failed to alter the total serum concentration of IgE antibody, despite being associated with significant elevations in liver

weight (Butala et al., 2004). Using a similarly high dose (50 mg) of DEHP delivered topically (with concurrent subcutaneous injection of the reference allergen OVA) we did not see any impact on the development of anti-OVA IgG1 or IgE antibody responses. However, in the same series of experiments a high dose of BBP (100 mg) significantly enhanced IgG1 anti-OVA antibody responses in the absence of any effect on IgE antibody production (Dearman et al., 2008, 2009). In interpreting these data it is important to appreciate that topical administration of chemicals to mice in the absence of occlusion will inevitably result in significant oral exposure as the result of grooming.

There is also some information available about the impact of inhaled phthalates on immune function in mice. Inhalation exposure to DEHP (up to 13 mg/m³) together with OVA had similar effects to that of simultaneous subcutaneous or ip administration of the two materials (Larsen et al., 2007). That is, increased IgG1 anti-OVA antibody levels were found in the absence of effects on IgE antibody production. Airway exposure to MEHP (a major metabolite of DEHP), had apparently identical effects to those seen with DEHP, but at considerably lower doses than those required for the parent compound (0.03 mg/m³) (Hansen et al., 2007). The authors calculated that, with respect to extrapolation to human airways, there was a margin-of-safety of approximately 50 (Larsen et al., 2007). They therefore drew the conclusion that “realistic” DEHP exposure levels likely to be encountered in the environment would not be expected to cause adjuvant effects in humans, or to result in allergic inflammation of the lung (Larsen et al., 2007).

Taken together, these experimental studies reveal that the route of administration of phthalate is of considerable importance, and appears to have a decisive influence on whether immunomodulatory effects are induced. Comparatively low doses of phthalates affect antibody parameters when administered only by routes of exposure (subcutaneous or ip) that do not reflect, and are not generally relevant for, opportunities for human contact with phthalates, except in the case of certain medical plastics. Although these are among the routes are favoured for experimental immunisation (Van Zijverden and Granum, 2000) and, in the case of subcutaneous administration, for the purposes of vaccination, neither are routes by which individuals would normally be expected to encounter phthalates in the domestic or occupational environment.

This interpretation is consistent with recent investigations in which potential routes of exposure to the most commonly used phthalates have been estimated employing scenario-based approaches (Wormuth et al., 2006). It was found that with respect to DEHP, DBP and DIBP, infants and toddlers receive the majority of their exposure orally through the ingestion of both dust and food, while in older children and in adults exposure is primarily through the diet. Similarly for DINP and DIDP, it is estimated that exposure of children results primarily from oral contact and ingestion (mouthing as well as ingestion of dust). For adults, exposure to DIDP is also largely by the oral route (from food and to a lesser extent dust), with dermal and inhalation pathways making smaller contributions. In contrast, in the case of DINP, it is the skin and inhalation pathways that make the largest contributions to adult exposure patterns, with lower levels encountered via oral ingestion. For BBP, oral ingestion is the most important route of exposure for children, with inhalation exposure assuming greater importance among adults (Wormuth et al., 2006). The data summarised above from studies in mice indicate clearly that the oral exposure to phthalates is substantially less effective at causing adjuvant-like effects than are either subcutaneous or ip exposures.

In reconciling effects seen in experimental models with potential hazards to human health it is relevant to consider the nature of adjuvant effects observed with phthalates in mice. Particularly in more recent studies, evaluations of effects on antibody production have been confined only to consideration of IgG1 responses.

As discussed previously, although IgG1 antibody production in the mouse is often used as a surrogate for IgE antibody responses, IgG1 is considerably less effective at inducing murine mast cell degranulation than is IgE, and in humans the class of antibody that mediates allergic and anaphylactic reactions is (almost exclusively) IgE (Lehrer and Vaughan, 1976; Ovary, 1982; Oshiba et al., 1996). With respect to human health a strong case can be made, therefore, that an induced elevation in mice of IgG1 antibody production, in the absence of effects on IgE antibody responses, is of lesser concern than would be a significant elevation in IgE levels.

One other aspect of the impact of phthalates on immune responses requires consideration. As summarised above, it is clear that under some circumstances, and at some doses, certain phthalates were reported to display what were described as “immunosuppressive” effects (inhibition of IgG1 and/or IgE antibody production), (Larsen et al., 2001a). This inhibitory activity was not exclusively a consequence of generalised systemic adverse effects as depression of antibody responses was not observed exclusively at maximal doses. One can, of course, speculate in general terms about variable dose-related effects of chemicals on the orchestration of immune responses, and in particular on immunoregulatory elements. It is nevertheless difficult to develop a plausible and coherent hypothesis to explain why in some circumstances certain phthalates appear to inhibit antibody production at lower doses, while augmenting the same responses at higher doses. It might be argued that at different dose levels such phthalates are acting selectively on distinct cellular vectors of immune function resulting in unexpected dose-related influences on immune responses. While it is not possible presently to discount such dose-selective effects, an alternative, and to our minds more plausible, explanation is that the readout of antibody production that has been used for evaluation of the potential impact on phthalates on the immune system is subject to natural fluctuations and perturbations that might not necessarily be treatment-related. If this is indeed the case then there is clearly need for some caution in defining the putative immunomodulatory properties of phthalates.

4.2.2. Effects of phthalates on other immune and inflammatory parameters *in vivo*

Although the majority of studies investigating the potential of phthalates to impact on immune function have focused on consideration of induced changes in antibody responses, less commonly, other readouts have been used. One such is the activity of discrete functional sub-populations of T lymphocytes that collectively dictate the quality and vigour of adaptive immune responses. A seminal observation, made over two decades ago, is that the induction and maintenance of IgE antibody responses is controlled by functional subpopulations of Th cells. It quickly became clear that the successful maturation of activated B lymphocytes into IgE producing cells, and a sustained IgE antibody response, requires the preferential development of the Th2 sub-population of Th cells. These cells produce an array of cytokines including interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13) that promote IgE antibody production and facilitate IgE-mediated allergic inflammation (Finkelman et al., 1988a,b; Mosmann et al., 1991). The other major functional sub-population of Th cells is designated Th1 cells. These cells display a different pattern of cytokine production, including, importantly, interferon- γ (IFN- γ) that antagonises the genesis of IgE antibody responses. Moreover, Th1 cells and their cytokine products serve to constrain and inhibit immediate-type allergic responses effected by IgE antibody (Finkelman et al., 1988a,b; Mosmann et al., 1991).

Against this background it is legitimate therefore to consider whether phthalates have any potential to impact on Th cell function, and in particular to influence or perturb the balance between Th1 and Th2 cells and their soluble cytokine products. Although relatively few studies of phthalates have sought to examine Th1- and

Th2-type responses as a function of differential cytokine expression, there is available some information.

Exposure of BALB/c strain mice to DEHP or to DINP (40–125 µg) by ip injection was reported to increase production of the Th2-type cytokine IL-4 by KLH-primed lymph node cells (LNC) (Lee et al., 2004). Some evidence for increased levels of IL-4 production by draining LNC associated with exposure to phthalates was also described by Maruyama et al. (2007). They sensitised CD-1 strain mice topically to the experimental contact allergen fluorescein isothiocyanate (FITC). The FITC was formulated either in a 1:1 mixture of acetone and various low molecular weight phthalate esters (DEP, DPP and DBP), or with acetone alone. Compared with mice that had been exposed to FITC in the absence of phthalates, those that had received phthalates topically were reported to produce increased amounts of IL-4 by draining LNC (Maruyama et al., 2007). The impact of phthalates on cytokine expression has also been investigated following inhalation exposure. In one series of experiments BALB/c strain mice were exposed by inhalation to OVA with or without DEHP. In this instance mice that received DEHP displayed not only elevated levels of IL-5 (a type 2 cytokine), but also increased amounts of the Th1 product IFN-γ (Larsen et al., 2007).

Finally, the impact of phthalates has been considered also following oral administration. Dietary exposure of rats to 12,000 ppm of DEHP was found to alter the balance of Th1/Th2 cytokines in the liver towards a preferential Th2 type phenotype. In the context of these experiments, however, the phthalate-induced Th2 response had an ameliorating effect on the tissue damaging Th1 response that was being provoked by the intraperitoneal administration of *Mycobacterium bovis* purified protein derivative (Badr et al., 2007).

It is important to acknowledge, however, that in other studies exposure of rodents to relatively high doses of DEHP and various other phthalates (BBP, DIHP and DINP) either by topical treatment, or via gavage, have failed to impact on cytokine expression (Butala et al., 2004; Piepenbrink et al., 2005). In the study by Piepenbrink et al. (2005), other immune parameters (lymphoid organ weights, thymus histology, antibody levels) were also found to be unaffected by oral administration of DEHP. In contrast, other authors have demonstrated that dietary exposure of C57BL/6 strain mice to relatively low levels of DEHP was immunosuppressive, resulting in a marked atrophy of both thymus and spleen (Yang et al., 2000).

In other investigations attention has focused instead on examination of whether exposure to phthalates is able to influence inflammatory processes. There are several studies which suggest that exposure to DEHP can enhance ongoing inflammatory responses. In a rat model of OVA-induced allergic asthma, gavage exposure to DEHP was associated with enhanced inflammatory cell infiltration in OVA-challenged airways (Yang et al., 2008). Similar general conclusions were drawn from the use of another rodent model, in this case of atopic dermatitis. Mice of NC/Nga strain develop signs and symptoms of atopic dermatitis. Using these animals it was found that ip exposure to DEHP exacerbated skin lesions induced by intradermal injection of house dust mite allergen. These changes were associated with increased expression of cutaneous chemokines such as macrophage inflammatory protein-1α (Takano et al., 2006). Furthermore, the same group reported that maternal ip exposure to DEHP during the neonatal (but not the fetal) period aggravated skin lesions in mite allergen-sensitised male offspring, but not in female offspring (Yanagisawa et al., 2008). The biological significance of these observations are as yet uncertain.

It can be concluded, therefore, that investigations of the effects of phthalates upon various immune and inflammatory responses in experimental animals have yielded conflicting, and somewhat variable, results ranging from potentiation of immune or inflammatory responses, to the absence of any effect, to inhibitory or immunosuppressive activity. Although the available evidence suggests that certain phthalates, when delivered at appropriate doses, and via

an appropriate route, can impact on immune and inflammatory function in rodents, it is clear that as yet no consistent pattern has emerged. There is clearly a case to be made for the design of more definitive animal studies that will allow development of a more detailed understanding of whether and to what extent, and under what conditions, certain phthalates are able to effect meaningful changes in immune function. In advance of that and access to more detailed information it is appropriate to question whether there are any other relevant data available.

4.2.3. Effects on the elicitation of clinical symptoms in asthmatics

There has been a single study in which the influence of inhalation exposure to phthalates on immune responses in humans has been examined (Deutschle et al., 2008). Patients allergic to house dust mite and healthy (non-allergic) controls were challenged (nose-only) with atmospheres containing low (0.41 mg/g) or high (2.09 mg/g) levels of DEHP. Clinical symptoms were scored, nasal secretions monitored for cytokine and chemokine expression, and a transcriptional analysis was performed on nasal biopsy samples. Nasal exposure to the dust was without effect on clinical symptoms for either group. House dust mite allergic patients, but not non-allergic individuals, responded to challenge with the DEHP_{low} dust with up-regulation of a number of markers of inflammation including IL-6 and granulocyte-colony stimulating factor. Exposure to the DEHP_{high} dust attenuated the response, down-regulating levels of inflammatory cytokines. These are interesting observations, but as the authors acknowledge, a short term exposure protocol (3 h) was used and it is therefore difficult to extrapolate from these data to normal environmental exposure patterns. Moreover, as the subjects were not challenged with house dust mite allergen alone, it was not possible in this study to distinguish between the effects due to low dose DEHP, and those due to house dust mite allergen alone.

4.2.4. In vitro effects

Finally, attempts have also been made to characterise *in vitro* the ability of various phthalates to impact on immune function. There are various investigations in which the impact of phthalates on primary immune cells, or immune cell lines, has been characterised in culture. In common with other aspects of toxicology, it is particularly important when using *in vitro* cell culture systems to demonstrate that any observed effects are not due to non-specific toxicity or other artefacts, and that biological significance is ascertained before extrapolation to definition of human health risks. In some *in vivo* experiments, inhibitory effects have been recorded, although in others immunostimulatory effects have been seen. There is some evidence that *in vitro* exposure of allergen-primed LNC to DEHP, or to DINP, was able to enhance the expression of IL-4, but was without effect on the type 1 cytokine IFN-γ (Lee et al., 2004). Similar phthalate-induced effects on IL-4 expression by a murine thymoma line (EL4) were reported by the same authors (Lee et al., 2004). In contrast, we failed to see any impact of phthalates on cytokine expression using a very similar experimental system (Dearman et al., 2010). In a rather different approach the impact of culture of the human monocytic cell line THP-1, or peripheral blood mononuclear cells (MNC) from allergic and non-allergic donors, with 6 different monophthalates on cytokine mRNA expression levels was examined (Glue et al., 2002). There was no effect of the monophthalates on cytokine expression by the THP-1 cells, or on MNC derived from non-allergic individuals, although increased expression of IL-4 in MNC from allergic individuals was reported for MNBP only (Glue et al., 2002). There are other reports that certain monophthalates promote the production by epithelial cell lines of inflammatory cytokines such as IL-6 and IL-8 (Jepsen et al., 2004). In these experiments, high doses of phthalate resulted in inhibition of cytokine production. Some phthalates, particularly

DEHP and MEHP, have been shown to augment histamine release by human basophils isolated from peripheral blood MNC; results interpreted as suggesting that phthalates may have an adjuvant effect on the elicitation of allergic responses (Glue et al., 2005). The physiological significance of this finding is, however, called into question by the observation that concentrations of DEHP reflective of likely human exposure levels failed to provoke clinical symptoms in allergic individuals (Deustchle et al., 2008).

A variety of other authors have demonstrated that culture of inflammatory cells such as macrophages with phthalates has various inhibitory effects, such as decreasing nitric oxide or tumour necrosis factor- α production, consistent with potentially immunosuppressive effects (Hong et al., 2004; Ohnishi et al., 2008). There is also literature that suggests that phthalates such as MEHP induce apoptosis in B cells (the cells that are responsible for the production of immunoglobulin), indicative of down-regulation of antibody responses (Schlezinger et al., 2004, 2007; Bissonnette et al., 2008). The general biological relevance of these observations is uncertain, as is their relationship, if any, to the immunomodulatory properties of phthalates.

5. Concluding comments

Although the increasing prevalence of atopic allergy and asthma that has been witnessed in 'westernised' countries since World War II now may be reaching a plateau, it remains important to understand the nature of environmental factors that have impacted on human health in this way. It is legitimate also that altered exposure to chemicals in general, or to specific classes of chemicals in particular, is considered as a possible contributing element. It is against this background that there has been interest in observations that exposure to certain phthalate esters, in either the workplace or home environment, may be associated with an increased risk of asthma and related respiratory symptoms. Intriguing as such associations are, it must be borne in mind that, as yet no causal relationship between phthalates and asthma has been established to the exclusion of other exposures, and that demonstration of dose-related correlations between phthalate exposure and respiratory symptoms has proven elusive. Moreover, even if there were found to exist some causal relationship between certain phthalates and asthma, it would not necessarily imply that phthalates are able to enhance the development of allergic sensitisation; the alternative argument being that exposure is associated with the exacerbation of respiratory symptoms in those who are already sensitised.

Despite these uncertainties about what conclusions can be drawn from the human experience, there has been some considerable interest in determining whether in experimental animals phthalates can influence the acquisition of allergic sensitisation to proteins. Those data are reviewed here, and although no coherent pattern emerges, it is possible to draw some general conclusions. The first of these is that the information from studies in mice suggest that certain phthalate esters are able to influence adaptive immune function (generally antibody responses) if exposure is effected at an appropriate dose and via an appropriate route. So, on this basis, there is a case to be made for certain phthalates displaying some intrinsic potential to modify immune responses under defined conditions. This is of interest, and it will be rewarding to establish the mechanisms through which such effects are mediated. However, in attempting to align the impact of phthalates on immune function in mice, with human atopic allergy and asthma, and with changes in the prevalence these diseases, a second important point needs to be made regarding the need for caution in interpreting these data. In this context the key considerations are that: (a) the doses at which effects are witnessed in mice are frequently very considerably higher than those likely to be experienced in work or domestic environments, (b) lower concentrations of

phthalates that perhaps better reflect likely human experience generally have impact on immune function only when administered by routes of exposure that are of less relevance for humans, (c) under those conditions in mice where treatment with phthalates does result in some change in antibody production, the changes induced can be either inhibitory or enhancing, and finally (d) even when exposure to phthalates does cause elevated antibody responses, this does not always embrace increases in IgE antibody.

Taken together, these data suggest that, although some phthalates may have an intrinsic ability to modify adaptive immune responses (under strictly defined conditions, and via mechanisms that have yet to be elucidated), there is no evidence to suggest that these chemicals have a consistent and proven ability to enhance allergic sensitisation under conditions of exposure that are relevant for human health. It is premature therefore to implicate phthalates as having contributed to the increasing prevalence of atopic allergy and asthma.

Conflict of interest

None.

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