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Does Lipophilicity Per Se Induce Adjuvant Effects? Methyl Palmitate as Model Substance Does Not Affect Ovalbumin Sensitization

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Anthropogenically introduced substances and pollutants are suspected to promote sensitization and development of allergic airway diseases, that is, acting as adjuvants. Lipophilicity may serve as an immunological warning signal, promoting adjuvant effects. Whether the lipophilicity of an inhaled compound induces immunomodulatory effects was investigated in a murine inhalation model with the highly lipophilic methyl palmitate (MP) as model substance. First, studies of acute effects following a 1-h exposure of up to 348 mg/m³ MP showed no effects on cell composition in bronchoalveolar lavage (BAL) or on lung function parameters. Thus, MP did not possess irritant or inflammatory properties, which may be a precursive stimulus for adjuvant effects. Second, mice were exposed to aerosols of MP, 6 or 323 mg/m³, for 1 h followed by a 20-min low-dose ovalbumin (OVA) inhalation. OVA only and OVA+Al(OH)₃ served as control groups. Exposures were performed 5 times/wk for 2 wk followed by a weekly exposure for 10 wk. Finally, the mice were challenged with a high-dose OVA aerosol for 3 consecutive days. Neither OVA-specific immunoglobulin (Ig) G1, IgE, or IgG2a production, nor inflammatory cells in BAL, nor respiratory patterns were significantly affected in the MP groups. The OVA+Al(OH)₃ group had a significantly higher IgG1 and IgE production, as well as higher eosinophil infiltration in the BAL fluid. These studies showed that effects of adjuvants are not necessarily due to their lipophilicity; that is, additional structural properties are required.

The increased prevalence of allergic respiratory diseases observed over the last decades (Lundback, 1998) has, among other things, been associated with anthropogenically introduced substances and pollutants, acting as adjuvants. Such adjuvants may promote the sensitization towards common aeroallergens. Thus, diesel exhaust particles (DEP) were found to promote the development of allergic asthma in epidemiological studies (Delfino, 2002) and to possess adjuvant effect in both animal (Heo et al., 2001; Steerenberg et al., 2003) and human experimental studies (Riedl et al., 2005).

Immune system recognition of structurally specific lipids is important for defense against infections, and lipids can play a role as self-antigens in autoimmune diseases (De Libero & Mori, 2005). Recently, lipophilicity by itself was suggested to possess immunostimulating properties (Seong & Matzinger, 2004). When lipophilic portions of a variety of both endogenous and exogenous molecules are presented to the innate immune system, they act as universal alarm signals, thus stimulating repair, remodeling, and immunity (Seong & Matzinger, 2004). Seong and Matzinger hypothesize that “hydrophobic portions can ... become damage-associated molecular patterns if exposed after damage or injury.” Several lipophilic formulations, such as incomplete Freund’s adjuvant (Chang et al., 1998) and liposomes (Copland et al., 2005), are potent adjuvants and several commercially available adjuvants are lipid based (Copland et al., 2005; Oda et al., 2004). Interestingly, DEP are covered with an outer layer of hydrocarbons (Figler et al., 1996), which were shown to possess adjuvant activity (Heo et al., 2001; Kadkhoda et al., 2005) due to promotion of cytokine, chemokine, and accessory molecule expression, and due to immunoglobulin (Ig) E production and promotion of IgE switching (Nel et al., 1998). However, highly lipophilic compounds may also cause immunosuppression (Colosio et al., 2005).

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Adjuvant effects may be due to different mechanisms (Cox & Coulter, 1997; Nel et al., 1998). For the DEP-associated hydrocarbons, an adjuvant effect may, for example, be due to formation of reactive oxygen species (Nel et al., 1998), which in addition to the adjuvant effect, may cause immunosuppression (Colosio et al., 2005). Thus, an adjuvant effect may appear as a balance between a toxic effect and a promotion of sensitization (Gupta et al., 1993). However, an adjuvant and a toxic effect may be caused by different mechanisms. For example, in a series of monophthalate esters from mono-*n*-butyl to mono-*iso*-decyl phthalate, the immunosuppressive effect increased with the size of the alcohol group, that is, with the lipophilicity, whereas the adjuvant effect followed a bell-shaped relation with maximum effect in the middle range of the studied alcohol chain lengths (Larsen et al., 2001a). Thus, a study of the relationship between lipophilicity and adjuvant effect has to be performed, where the influence of toxic effects is equal, minimal, or absent.

Many anthropogenically introduced substances are lipophilic. This raises a general question: Does the lipophilicity of a chemical substance by itself induce an adjuvant effect? It also raises the question of which is the relevant exposure route. An increase in allergic airway diseases was reported for a variety of common aeroallergens (Nielsen et al., 2002), suggesting that anthropogenic substances may exert adjuvant effects via inhalation. Therefore, risk assessment should be based on the relevant human exposure route and thus should be based on either epidemiological studies (Nielsen et al., 2005) or inhalation studies in animals, where animals are sensitized exclusively via the airways (Clausen et al., 2003).

Phthalates are lipophilic substances, and di-(2-ethylhexyl) phthalate (DEHP) was reported to be associated with the development of asthmatic reactions in epidemiological studies (Jaakkola et al., 1999; Oie et al., 1997). Moreover, DEHP demonstrated adjuvant effects in a screening study in mice that used parenteral administration of DEHP together with the model allergen ovalbumin (OVA) (Larsen et al., 2001b). DEHP has low toxicity by inhalation (Klimisch et al., 1992).

To test whether adjuvant effects can be ascribed to lipophilicity, methyl palmitate (MP) was selected as a model of lipophilic substances and tested in a murine inhalation sensitization model (Clausen et al., 2003). MP and DEHP possess comparable lipophilic properties [log octanol/water partition coefficients of 7.38 (Krop et al., 1997) and 7.5 (Staples et al., 1997), respectively]. First, acute effects on the airways were studied to assess irritant and inflammatory properties, which may promote adjuvant activity (Granum & Lovik, 2002). MP had a low toxicity, as was the case with DEHP. Second, mice were repeatedly exposed to aerosols of MP and OVA for up to 3 months and the adjuvant potential was assessed from the production of OVA-specific antibodies in serum and inflammation in the airways. Under the conditions of the test, MP showed no adjuvant effects.

MATERIALS AND METHODS

Animals

Inbred female BALB/cJ mice were purchased from Taconics M & B, Ry, Denmark, at age 5–6 wk, weighing 20.5 ± 1.8 g (mean $\pm$ SD) at the beginning of the experiments. Mice were housed 5 animals together in polypropylene cages ($380 \times 220 \times 150$ mm) with pinewood sawdust bedding (Lignocel S8, Brogaarden, Denmark), gnaw sticks, and cardboard tubes. The photoperiod was from 6 a.m. to 6 p.m., and the temperature and relative humidity in the housing room were 21–23°C and 42–70%, respectively. Cages were sanitized twice weekly and OVA-free food (Altromin number 1324, Altromin, Lage, Germany) and tap water were available ad libitum. Mice were acclimatized for 2 wk before the start of the experiment. For the adjuvant study, mice were conditioned to experimental procedures by placing them in head-out plethysmographs (see below) for 15 min each day for 5 consecutive days, 1 wk before starting exposures. Each mouse was marked on the tail with a felt-tip pen. Body weights were followed during the experiment and used as a general health indicator. All studies were performed in accordance with procedures approved by the Animal Experiment Inspectorate, Denmark.

Generation of Test Atmospheres and Particle Characterization

MP (CAS 112-39-0, purity >97%, Sigma-Aldrich, Germany) was dissolved in acetone (CAS 67-64-1, purity >99.5%, Sigma-Aldrich, Germany) at concentrations of 8% and 75% w/v. A Pitt number 1 aerosol generator (Wong & Alarie, 1982) coupled to an infusion pump (New England Medical Instruments Inc., MA, USA) aerosolized the MP solutions into a glass/stainless steel exposure chamber (Clausen et al., 2003) to concentrations of 6 ± 2 , 8 ± 1 , 58 ± 16 , 323 ± 119 , and 348 ± 107 mg/m³. The acetone concentration did not exceed 1900 ppm, which previously was shown not to have an irritant effect on the airways (Larsen et al., 2004). To keep the high concentrations of MP in a liquid state (MP melting point: 26°C), a fan was used to heat the MP solution and the exposure chamber's inlet air. Temperatures inside the exposure chamber never exceeded 30°C. OVA (grade V, Sigma-Aldrich, Germany) was dissolved in pyrogen-free 0.9% NaCl to a feeding solution of 25 mg/L or a 1% w/v solution. The concentration of the Al(OH)₃ adjuvant (Alhydrogel, Brenntag Biosector, Denmark) in the 25-mg/L OVA solution (OVA+Al(OH)₃) was 0.5% w/v. The OVA and OVA+Al(OH)₃ aerosols were generated by a PARI LC STAR nebulizer (PARI GmbH, Germany).

Air samples were collected from the breathing zone of the mice, and exposure concentrations of MP, OVA (C_{OVA}), and OVA+Al(OH)₃ were measured gravimetrically (Clausen

et al., 2003). It was assumed that OVA, $\text{Al}(\text{OH})_3$, and NaCl constituted the same fractions in the aerosols collected on filter as in the feeding solutions. The aerodynamic diameter distribution of different OVA and MP solutions was determined in separate experiments using a Marple personal cascade impactor, model 296, with a model 290-IA inline adaptor (Sierra Instruments, Graseby) (Table 1) (Clausen et al., 2003). Deposition fractions of monodisperse aerosols with mass aerodynamic diameter 0.3, 1.1, and ≥ 3.5 μm from Raabe et al. (1988) were accepted to represent deposition fractions of particles <0.6 , $0.6\text{--}1.6$, and ≥ 1.6 μm in this study. The inhaled dose was calculated from: $C_{\text{OVA}} \times 245 \text{ breaths/min} \times 0.15 \text{ ml (tidal volume)} \times \text{min (length of exposure period)}$. The respiratory parameters were from Clausen et al. (2003) and the estimates of deposition were performed as in Clausen et al. (2003), as the same equipment and mouse strain were used.

Protocol for Study of Acute Effects of Methyl Palmitate

For studies of acute respiratory effects of MP, mice weighing 20.8 ± 1.9 g were exposed for 15 min to laboratory air to obtain baseline values for each respiratory parameter for each mouse. This was followed by a 60-min exposure to different concentrations of MP or to acetone (1900 ppm) as the control group. The effect of 30°C heated acetone was also assessed. Parts of the data from the acute study of the 8 mg/m^3 MP and acetone exposure were derived from the d 1 exposure of the sensitization study (described later). Bronchoalveolar lavage (BAL) and total and differential cell counts were performed at different time points following exposure to 58 and 348 mg/m^3 MP.

Protocol for Study of Adjuvant Effect of Methyl Palmitate

Groups of 9–10 mice (weight at d 1: 19.9 ± 1.4 g) were exposed to either MP or acetone (control group) for 1 h, followed by 20 min of exposure to a nebulized OVA solution (25 mg/L). Mean MP air concentrations were 6 ± 2 (low MP) or 323 ± 119 (high MP) mg/m^3 . A positive control group was exposed to acetone for 1 h, followed by OVA + $\text{Al}(\text{OH})_3$.

TABLE 1
Mass Fractions (%) of Aerosol Particles

Aerosol	Diameter (μm)		
	$0 < d < 0.6$	$0.6 \leq d \leq 1.6$	$1.6 < d$
OVA (25 mg/L)	13	39	48
OVA (1%)	36	26	38
Low MP	3	28	69
High MP	5	41	54

Mean air concentrations are shown in Table 2. Aerosol exposures were repeated for 5 consecutive days/wk for 2 wk, followed by 1 weekly exposure for 10 wk. The day after the weekly exposure, $100 \mu\text{l}$ blood was collected from the tail vein for analysis of OVA-specific IgG1 and, when enough blood was available, also for OVA-specific IgE antibodies. One week after the 10th weekly exposure, tail vein blood was collected and all groups were challenged to a solution of 1% OVA for 20 min for 3 consecutive days. The day following the last challenge, mice were bled by heart puncture and blood collected for the analysis of OVA-specific IgG1, IgE, and IgG2a antibodies. BAL was performed, as well as total and differential cell counts. Respiratory parameters were measured on d 1 and 10, during the weekly exposures, and the 3 challenges. On these days, mice were exposed to laboratory air for 15 min before the regular exposure regimens to collect baseline respiratory values as described for the acute study.

Collection of Respiratory Parameters

Groups of up to 10 mice were placed in body plethysmographs in the exposure chamber for head-only exposure. Respiratory parameters were monitored by means of the Notocord Hem data acquisition software (Notocord Systems SA, France), which for example calculates respiratory rate (f , breaths/min), time from the end of inspiration to the beginning of expiration, termed time of brake (TB, ms), time from the end of expiration until the beginning of next inspiration, termed time of pause (TP, ms), tidal volume (VT, ml), and mid-expiratory flow rate (VD, ml/s). Settings of the data acquisition modules were according to Larsen et al. (2004). These parameters were used to assess upper and lower airway irritation, which can be assessed from elongation of TB and TP, respectively. Airflow limitation is observed as a decrease in the ratio VD/VT (VD corrected for change in VT). Generally, respiratory effects are reflected by changes in f (cf. Nielsen et al., 1999, for comprehensive descriptions).

In the acute study, the mean value of a 60-min period was presented as percent mean baseline value to estimate effects on respiratory parameters. As mice in the acute studies were not adapted to restraint, this could occasionally affect their breathing patterns disproportionately. Consequently, an animal was excluded if it had a respiratory parameter that deviated from the period mean ± 3 SD obtained from the remaining animals during evaluation of the respiratory data. This was done for 3 of 26 animals and 3 of 20 animals in the 58- and 348-mg/m^3 exposure groups, respectively.

During the adjuvant study, effects on f were determined for the 1-h exposure to acetone or MP and for the last 5 min of the OVA exposure as percent baseline value. In this way a curve of "exposure time point" versus " f as % baseline" was plotted for each animal. The mean area under the curve (AUC) was calculated for each animal and evaluated statistically.

TABLE 2
Deposition of Ovalbumin (OVA) or Methyl Palmitate (MP) Aerosols

Concentration in feeding solution	Number of exposures ^a	Air concentration during exposure ^b	Cumulated doses (μg) of OVA or MP			
			Inhaled	Deposited in lungs	Deposited in upper respiratory tract	Deposited in gastrointestinal tract
25 mg/L OVA	20	0.14 (0.02)	1.7	0.4	0.5	0.8
25 mg/L OVA + 0.5% Al(OH) ₃	20	0.17 (0.04) ^c	1.9	0.4	0.6	0.9
1% OVA solution	3	49.9 (9.5)	86	31	22	33
8% w/v MP (low MP)	20	6.2 (2.4)	240	28	91	121
75% w/v MP (high MP)	20	323 (119)	11569	1813	3811	5945

^aExposures were for 60 min/d for MP and 20 min/day for OVA and were performed daily for 2×5 d and then weekly for 10 weeks. A 20-min challenge with a 1% OVA solution was performed on 3 consecutive days 1 wk after the last weekly exposure.

^bAir concentration is in mg/m^3 and values are mean and SD in parentheses.

^cAir concentration of OVA. The mean air concentration of Al(OH)₃ was $21 \text{ mg}/\text{m}^3$.

Measurement of Ova-Specific Immunoglobulins

OVA-Specific IgG1, IgE, and IgG2a antibodies in serum were measured by enzyme-linked immunosorbent assay (ELISA) according to Clausen et al. (2003). The amount of antibodies was compared with pooled standard serum and expressed in arbitrary units (AU).

Bronchoalveolar Lavage

The same person performed BAL by flushing the lungs 4 times with 0.8 ml saline. The recovered BAL fluids from each mouse were pooled and centrifuged ($500 \times g$, 4°C , 10 min). The cell pellet was used for determination of total cell numbers (resuspended in 100 μl phosphate-buffered saline, PBS, containing heparin at 20 IE/ml) and serum albumin at 0.003%) and for producing cytospin preparations ($1000 \times g$, 4 min, RT). Slides were stained with May-Grünwald/Giemsa, and for each slide, 200 cells were differentiated. Accordingly, the total number of each cell type was calculated.

Statistics

Statistical analyses were performed by the SAS System version 8.02 if not otherwise mentioned. Respiratory data from the acute exposures were compared by median values and corresponding nonparametric 95% confidence intervals (CI) calculated by the Wilcoxon sign test (MINITAB[®] Release 14.1). In the sensitization study, data from the 4 experimental groups were compared by means of the Kruskal–Wallis test. Differences from the OVA group were analyzed by the Mann–Whitney *U*-test. Differences in responder fractions were tested by Fisher's exact test. Immunoglobulin levels before and after the 3 challenges were compared by the Wilcoxon matched pair signed rank test. The Spearman correlation analysis was performed to reveal dose-dependent

effects of MP exposure in the OVA and two MP groups. Results were considered significant if $p < .05$.

RESULTS

Acute Effects of Methyl Palmitate

Respiratory parameters were followed during the acute exposure to 8, 58, or $348 \text{ mg}/\text{m}^3$ MP for 1 h. Mean baseline values of all tested animals were; *f*, 238 ± 26 breaths/min; TB, 28 ± 9 ms; TP, 39 ± 6 ms; VT, 0.17 ± 0.03 ml; VD, 1.74 ± 0.38 ml/s. This was in agreement with results from a previous study (Nielsen et al., 1999). Exposure effects on *f*, TB, TP, VT, VD, and VD/VT for each exposure group are shown in Table 3 as percent baseline values. Comparing the control (acetone) groups, it is seen that heat exerted a pronounced effect on VT and correspondingly produced a decrease in VD, indicating that the 58- and $348\text{-mg}/\text{m}^3$ MP exposure groups should be compared to the similarly heated acetone control group. If so, no exposure-related effect on *f*, TB, TP, VT, or VD was apparent following acute MP exposure.

The inflammatory effect of MP exposure was evaluated from leukocytes in BAL fluid, which was collected at different time points following acute exposure to 58 and $348 \text{ mg}/\text{m}^3$ MP. In the $348\text{-mg}/\text{m}^3$ MP group, no time-dependent effect was apparent (Figure 1). Also, cell numbers were similar to those in an acetone control group in a previous study (Larsen et al., 2004). The cell numbers in the $58\text{-mg}/\text{m}^3$ MP group were equal to the numbers in the $348\text{-mg}/\text{m}^3$ MP group (data not shown). Overall, neither respiratory nor inflammatory effects of a 1-h exposure with up to $348 \text{ mg}/\text{m}^3$ MP were observed.

Adjuvant Effects of Methyl Palmitate

Mouse weights. The weight gain during the study did not differ significantly among the 4 groups of mice, and the growth

TABLE 3
Effects on Respiratory Parameters During Acute Exposure to Acetone and Methyl Palmitate (MP)

Exposure ^a	MP ^b	Heat	<i>n</i> ^c	<i>f</i> ^d	TB ^d	TP ^d	VT ^d	VD ^d	VD/VT ^d
Acetone	0	—	35	98 (96.5–100)	106 (102.5–121.5)	107 (103–110.5)	95 (94–96.5)	95 (91.5–96.5)	100 (96.5–101)
Acetone	0	+	12	89 (87–92)	140 (120–182.5)	111 (102.5–120.5)	77 (73.5–81.5)	70 (67–73)	90 (87–94)
MP	8	—	19	95 (92–96)	115 (107.5–136)	102 (97–108)	90 (87.5–91.5)	85 (81–88)	95 (92–98)
MP	58	+	23	91 (87.5–97.5)	102 (95–115)	103 (95.5–106)	83 (79.5–86.5)	79 (72.5–81)	91 (87.5–97)
MP	348	+	17	94 (89–100)	113 (102–127.5)	98 (93.5–114)	75 (72–79.5)	73 (66–79)	97 (88.5–103.5)

Note. Groups of mice were exposed for 60 min to acetone or different concentrations of MP with or without heat. Respiratory parameters were monitored during a baseline period of 15 min and during the 60 min MP exposure period.

^aAcetone exposure concentration was 1900 ppm. MP was dissolved in acetone and the corresponding acetone concentration was $\leq$ 1900 ppm.

^bMP exposure concentration (mg/m³).

^cNumber of animals.

^dRespiratory rate (*f*), time of brake (TB), time of pause (TP), tidal volume (VT), tidal midexpiratory flow (VD) and the ratio VD/VT were calculated as percent baseline value for each mouse. Values are median of the group and nonparametric 95% confidence intervals.

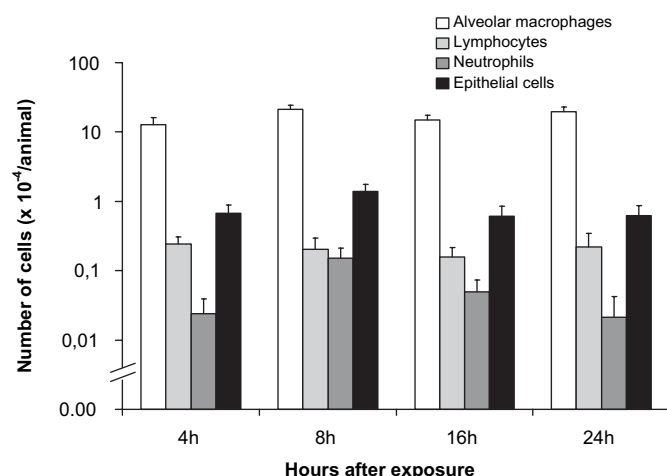


FIG. 1. Time-dependent effects on cell composition in BAL of mice exposed for 1 h to 348 mg/m³ MP. Lungs were lavaged at different time points following exposure and differential and total cell counts performed. No eosinophils were detected. Mean and 95% confidence limits of 5 animals are shown. The y axis is broken to include undetectable (0) levels.

curves were in concordance with a standard growth curve of female BALB/cJ mice obtained from the breeding center (data not shown).

Characterization of aerosols. The mass fractions of OVA and two MP aerosols were characterized (Table 1) to estimate total deposition of OVA and MP in the respiratory and gastrointestinal tracts (Table 2) during the sensitization study. Comparable doses of OVA were deposited in mice exposed to either OVA or OVA + Al(OH)₃ with approximately half of the

inhaled OVA deposited in the airways (Table 2). In contrast to the estimated, cumulated low dose inhaled during the sensitizing exposures (<2 µg), the 3 challenges gave rise to high amounts of OVA reaching the lungs (31 µg). The particle size of the low and high MP aerosols (Table 1) enabled deposition in the entire respiratory tract (Table 2).

OVA-specific antibody production. During the 10 weekly aerosol exposures, only a few animals responded with OVA-specific IgG1 and IgE antibody production in the OVA, OVA + low MP, and OVA + high MP groups and only at low levels (Table 4 and data not shown). In the positive control group, OVA + Al(OH)₃, the number of responders steadily increased after the second weekly exposure, and these animals continuously produced higher levels of IgG1 (Figure 2). IgG1 production preceded production of OVA-specific IgE (Figure 2), and IgE was only detected in animals also producing IgG1 (data not shown).

After 10 weekly exposures and before the 3 challenges, the number of animals producing IgG1 and IgE antibodies was significantly higher in the OVA + Al(OH)₃ group compared to the OVA group (Table 4). Accordingly, the median antibody production in the OVA + Al(OH)₃ group was significantly higher compared to the OVA group. The responder fractions and median antibody levels of the OVA group and the two MP groups were comparable (Table 4).

After the 3 challenges, the IgG1 levels in the responding animals in the OVA + Al(OH)₃ group fell markedly by approximately 30% from a mean of 16,900 AU to 11,730 AU. Similarly, IgE levels fell significantly by approximately 65% from a mean of 276 AU to 96 AU. OVA-specific IgG2a production, which was measured following the 3 challenges only, was

TABLE 4

Effects of Repeated Exposures on the Number of Responders and Median Antibody Production Before Challenges and on Respiratory Frequency

Aerosol exposure	OVA-specific IgG1		OVA-specific IgE		<i>f</i> during exposures	
	Number of responders/ number of samples	Median ^a	Number of responders/ number of samples	Median ^a	Acetone or MP ^b	OVA ^b
OVA	2/10	0 (0–0)	0/7	0 (0–0)	7232 ± 191	8453 ± 165
OVA + low MP	1/10	0 (0–0)	1/9	0 (0–0)	7345 ± 325	8102 ± 401
OVA + high MP	1/9	0 (0–0)	1/8	0 (0–0)	7635 ± 393	7554 ± 310
OVA + Al(OH) ₃	8/10*	1336 (350–10177)*	7/8*	91 (32–128)*	7558 ± 350	8346 ± 411

Note. Exposures, 1 h of acetone or MP followed by 20 min of OVA, were performed daily for 2 × 5 days and weekly for 10 wk. One week later, a 20-min challenge to a 1% OVA solution was performed for 3 consecutive days. Median arbitrary units (AU) and the number of responders were analysed from the blood sampling 6 d after the last weekly exposure and immediately before the 3 challenges. During all exposures, the mean respiratory rate, *f*, was calculated either for the 1-h exposure to acetone or MP or for the last 5 min of the OVA exposure as percent baseline value (cf. Materials and Methods). In this way a curve of “exposure time point” vs. “percent baseline” was plotted. The area under the curve (AUC) was calculated for each mouse. The number of animals in each group was 10 except for the OVA + high MP group, where *n* = 9. Asterisk indicates significant at *p* < .05 relative to the OVA group.

^aValues are AU and 25–75% percentiles are presented in parentheses.

^bGroup mean ± SD of the AUC is presented.

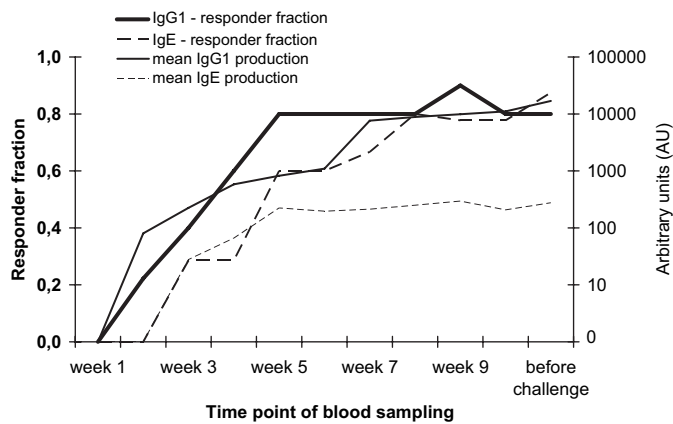


FIG. 2. Responder fraction and development of OVA-specific IgG1 and IgE antibodies in the OVA+Al(OH)₃ group. Mice were exposed to OVA+Al(OH)₃ for 2 × 5 d and once a week for 10 wk and finally received 3 1% OVA challenges. The responder fraction of animals producing OVA-specific antibodies and mean antibody levels was followed after the weekly exposures and before the last 3 aerosol challenges. The secondary y axis is broken to include undetectable (0) levels.

found in one or two animals in each of the 4 experimental groups and only at levels close to the detection limit (data not shown).

Effect of Repeated Aerosol Exposures on Composition of Cells in Bronchoalveolar Lavage

BAL was performed following 3 aerosol challenges to a 1% OVA solution at termination of the experiment. Inflammatory

reactions in the lungs are reflected by an increase in inflammatory cells in BAL (Brewer et al., 1999), that is, leukocytes, neutrophils, macrophages, and eosinophils. Correlation analysis showed no dose-dependent effect of MP exposure on total inflammatory cell numbers in BAL fluid. Comparing the OVA and OVA+Al(OH)₃ groups showed a significantly increased number of eosinophils in the adjuvant group (Figure 3). Regarding dose-dependent effects on macrophage numbers, there was a significant, positive relationship, but only a significantly increased number of alveolar macrophages in the OVA+high MP group compared to the OVA group. In addition, the OVA+Al(OH)₃ group had a significantly increased number of macrophages compared to the OVA group.

Respiratory Effects

On d 1 and 10, during the subsequent 10 weekly exposures, and the 3 OVA challenges, respiratory parameters were collected during aerosol exposures and analyzed as percent baseline value for each mouse. The repeated exposures to acetone or MP had no significant effect on *f* when comparing the AUC for the 4 groups (Table 4). Decreases or increases in *f* may be used as general indicators of effects on the respiratory tract. Consistently, no effects were observed on other respiratory parameters (data not shown). The observed OVA sensitization of the mice in the OVA+Al(OH)₃ group did not result in airway hypersensitivity responses after exposures or challenges to OVA as seen from comparisons of the AUC values from observations of *f* (Table 4). Effect on *f* was calculated as the mean value for the last 5 min of OVA exposure, as

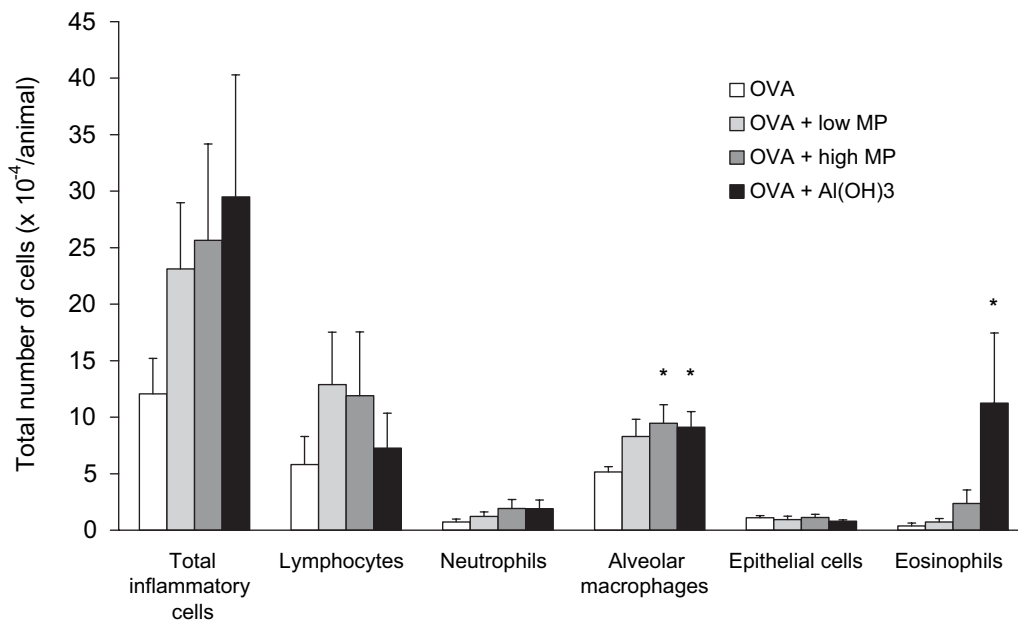


FIG. 3. Effects on BAL cell composition. Mice were exposed for 2 × 5 d and once a week for 10 wk. Total and differential cell counts were performed following 3 final challenges to a 1% OVA solution. Mean and SEM (error bars) are presented. Differences from the OVA group is indicated by asterisk for significance at *p* < .05.

a hypersensitivity response presumably would be most prominent at this time point. No effect related to the OVA exposure was observed on other respiratory parameters (data not shown).

DISCUSSION

Endogenous and exogenous lipophilic (macro)molecules may stimulate the immune system by activating pattern-recognition receptors, which may result in inflammation (Seong & Matzinger, 2004) and then be a precursive stimulus for adjuvant effects (Granum & Lovik, 2002). However, the airway secretion contains phospholipids and surfactant proteins that may capture inhaled lipophilic compounds in micelles and prevent their presentation to cells of the immune system, counteracting the development of inflammation (Pison et al., 1994). Hence, this raises the question of whether high lipophilicity of chemicals per se would induce adjuvant effects following airway exposure.

A previously developed inhalation model (Clausen et al., 2003) with low-dose OVA exposures was used for the study of adjuvant effects attributed to lipophilicity. The low-dose OVA regime minimizes development of tolerance (Clausen et al., 2003; Seymour et al., 1998), which is the predominant respiratory effect towards inhaled aeroallergens (Swirski et al., 2002). Aerosols of the adjuvant, $\text{Al}(\text{OH})_3$, were able to overcome tolerance and promote production of OVA-specific IgE and IgG1 antibodies (Clausen et al., 2003), which are both stimulated by $\text{T}_\text{H}2$ cytokines (DeKruyff et al., 1993). IgE antibodies are a key factor in the development of allergic asthma (Geha et al., 2003). In the present study, the number of weekly exposures was increased from 6 (Clausen et al., 2003) to 10 exposures followed by 3 high-dose OVA challenges. Both IgE and IgG1 levels declined significantly following the 3 challenges, probably due to antibodies binding to OVA in tissues or in blood. Nevertheless, the exposures increased eosinophil inflammation in the OVA + $\text{Al}(\text{OH})_3$ group — another characteristic of allergic asthma. However, the OVA + $\text{Al}(\text{OH})_3$ exposures did not produce bronchoconstriction or epithelial shedding, which are other findings after allergen exposures in asthma patients. Overall, the model used was able to detect adjuvant effect from endpoints relevant for allergic airway diseases (Nielsen et al., 2005) and therefore was applicable for risk assessment of adjuvant effects due to airborne substances.

The ester MP was selected as model substance. It has a high lipophilicity, approximately equal to that of the diester, DEHP. As the lungs contain esterases (Frederick et al., 2002), it is presumed that MP at least partly is hydrolyzed to methanol and palmitic acid/palmitate, which was the case with a pro-drug ester from a glucocorticosteroid and palmitate in the nasal mucosa (Ahlstrom-Emanuelsson et al., 2004). At the highest MP test concentration in our study, 348 mg/m^3 , a complete hydrolysis would correspond to about 41 mg/m^3 of methanol. This concentration is of no toxicological concern as the threshold limit value is 262 mg/m^3 (ACGIH, 2004). If,

however, palmitic acid accumulates in the lungs, it may activate the peroxisomal proliferator-activated receptor (PPAR) α (Kota et al., 2005), which was associated with both pro- and anti-inflammatory effects (Clark, 2002). Overall, MP was selected for its high lipophilicity, comparable to the lipophilicity of DEHP. Further, the degradation products of both MP and DEHP are PPAR α agonists (Kota et al., 2005; Rose et al., 1999).

An inflammatory effect may in itself promote sensitization (Granum & Lovik, 2002). Thus, inflammation may recruit dendritic cells to the inflammatory site in the lungs, increasing their maturation and migration to draining lymph nodes, where they may prime naive T cells (Lambrecht, 2005; Upham, 2003), as the first steps in the sensitization process (Corry & Kheradmand, 1999). Here, the highly lipophilic substance MP did not induce inflammatory or lung function adverse effects after acute exposures of up to 348 mg/m^3 MP. Palmitic acid is a lipophilic carboxylic acid with surface-active properties, as is mono-2-ethylhexyl phthalate (MEHP), the main metabolite of DEHP. MEHP had a no-observed-adverse-effect level of 0.3 mg/m^3 for airway irritation (Larsen et al., 2004). Due to the structural similarity to MEHP and the common surface-active properties, palmitic acid is also expected to be an airway irritant. The present observations indicate that little palmitic acid may have accumulated in the lungs. Similarly, in an unpublished study, it was found that no irritant effects of DEHP occurred at airborne concentrations up to 290 mg/m^3 (Larsen et al., unpublished observations), which may be explained by a low rate of hydrolysis of DEHP to MEHP in the lungs (Ito et al., 2005).

Repeated inhalation of MP followed by OVA did not affect OVA-specific IgG1, IgE, or IgG2a antibody production. Further, no long-term effects were observed in lung function studies. Consistently, no inflammation was observed in BAL, as the numbers of eosinophils and neutrophils were unaffected. Epithelial cells comprised up to 10% of the BAL cells. As the number was similar in all acute- and repeated-exposure groups, this demonstrates the absence of an exposure-dependent effect. Both the high MP and the $\text{Al}(\text{OH})_3$ exposure increased the number of macrophages in the airways, which probably could be ascribed to a particulate effect of the aerosols, as they are delivered to the respiratory tract as oil and gel droplets, respectively. Macrophages are scavengers of particulate material reaching the lung, and increases in macrophage numbers were found after particulate challenges in rats (Donaldson & Tran, 2002) and rabbits (Jones et al., 2002). This is further supported by the dose-dependent increase in macrophage numbers. Lung macrophages generally inhibit $\text{T}_\text{H}2$ and promote $\text{T}_\text{H}1$ responses in the lungs (Tang et al., 2001). Nevertheless, the increase in macrophage numbers after repeated MP exposures presumably did not affect the immune response, as a rise in macrophage numbers also was observed in the OVA + $\text{Al}(\text{OH})_3$ group, which developed both OVA-specific IgE and IgG1 antibodies. Furthermore, MP was used to inhibit liver macrophages

(Rose et al., 1997). If such a process had occurred in the lungs, this should have promoted a T_H2 response (Tang et al., 2001) and increased OVA-specific IgE and IgG1, which was not observed. This suggests that MP did not exert effects through macrophage-related mechanisms. Furthermore, although the degree of hydrolysis of MP in the lungs is unknown, the lack of airway irritation suggests that accumulation of palmitic acid, and thus activation of PPAR α receptors with immunomodulating effects did not appear to occur. In conclusion, high lipophilicity by itself need not give rise to danger signals or immunostimulatory effects, even after repeated exposures at concentrations far above those to which humans are exposed.

The question can be raised of whether the MP exposure was able to affect the immune response, as MP and OVA was administered successively in contrast to OVA and Al(OH) $_3$. The limitation of this protocol is that the OVA + Al(OH) $_3$ group serves as an assay control and not as a control to the MP exposure. The half-life of OVA in lungs is approximately 6 h, when calculated from data in Seymour et al. (2002). The half-life of removal of dipalmitoylphosphatidylcholine from the lungs was calculated to be approximately 3 h from Fisher et al. (2005). Assuming a similar half-life for MP and thus that MP is not degraded very rapidly, it is apparent that MP is present in the lungs during the OVA exposure. Other studies in both mice and humans have used successive administration of adjuvant and allergen and were able to show adjuvant effects on the T_H2 response (Granum et al., 2001; Riedl et al., 2005; Rumold et al., 2001). This suggests that the successive exposure to MP and OVA cannot account for the lack of an immune response.

In intranasally exposed mice, it has been shown that LPS contamination at low doses (0.1 μ g) stimulates a T_H2 response as seen from increased IgE levels and airway eosinophilia (Eisenbarth et al., 2002). In contrast, a higher dose of 100 μ g stimulates T_H1 responses and triggers neutrophil influx to the lungs (Eisenbarth et al., 2002). Neutrophil influx was shown to be maximal 24 h after lipopolysaccharide (LPS) administration (Hirano, 1997; Szarka et al., 1997). In the OVA and the two MP groups, no influx of eosinophils or neutrophils occurred in BAL 24 h after the last challenge and no stimulation of either T_H1 or T_H2 antibody production was observed. Overall, this suggests that LPS contamination did not play a significant modulating role, hampering the detection of a possible adjuvant effect of MP. Moreover, a clear adjuvant effect was seen due to the Al(OH) $_3$ exposures in the positive control group.

In conclusion, an inhalation mouse model was used that allows detection of adjuvant effects on antibody production and eosinophilic airway inflammation. Here, it was used to study the adjuvant effect of airborne MP, a model compound with prominent lipophilic properties. No adjuvant effects were observed, which suggests that the mechanisms of adjuvants are not necessarily due to their lipophilicity. Further studies are needed to elucidate the effects of other structural properties of adjuvants.

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