

Development of sensitisation or tolerance following repeated OVA inhalation in BALB/cJ mice. Dose-dependency and modulation by the Al(OH)₃ adjuvant

Susanne Knoth Clausen^a, Mette Bergqvist^a, Lars K. Poulsen^b,
Otto Melchior Poulsen^a, Gunnar Damgård Nielsen^{a,*}

^a Department of Chemical Working Environment, National Institute of Occupational Health, Lersø Parkallé 105, DK-2100 Copenhagen, Denmark

^b Laboratory of Medical Allergology, National University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark

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Abstract

Anthropogenically induced exposures may, due to their adjuvant effect, promote development of sensitisation to commonly occurring aeroallergens. No generally accepted model exists for determination of adjuvant effect of airborne substances. Therefore, BALB/cJ mice were exposed for 10 consecutive days with ovalbumin (OVA) solution, 25 mg/l–10 g/l (0.0025–1%) for 20 min/day, with and without the Al(OH)₃ adjuvant (0.5%). Four days after the last aerosol exposure, no OVA specific IgE and only low IgG1 were produced. Subsequent parenteral OVA administration showed that the 10 g/l solution induced full tolerance of the IgE response, whereas only partial tolerance was apparent with 25 mg/l OVA. The Al(OH)₃ adjuvant counteracted development of tolerance that was fully prevented at the 25 mg/l OVA concentration. Development of IgG1 was increased in a concentration-dependent manner with 500 mg/l–10 g/l OVA. No increase occurred at the 25 mg/l level, but addition of Al(OH)₃ increased IgG1 production to the same level as the higher OVA concentrations. Concentrations from 1.25 mg/l to 10 g/l OVA were studied with ten exposures followed by once-weekly aerosol exposure for up to 6 weeks. In the range from 1.25 mg/l to 10 g/l, IgE production was time- and concentration-dependent. Both the IgE and IgG1 production were markedly promoted by Al(OH)₃. However, with aerosol exposures, the IgE antibody productions were not sufficient to increase the level of inflammatory cells in broncho-alveolar lavage fluid. Overall, this study showed that airborne Al(OH)₃ was able to counteract tolerance and increase specific IgE and IgG1 production.

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Abbreviations: Al(OH)₃, aluminium hydroxide; OVA, ovalbumin; PBS, phosphate buffered saline; a.u., arbitrary units; BAL, bronchoalveolar lavage; RT, room temperature.

* Corresponding author. Tel.: +45-39-16-52-41

E-mail address: gdn@ami.dk (G.D. Nielsen).

1. Introduction

The prevalence of respiratory diseases, asthma and rhinitis, are increasing in the Westernised countries. It is primarily the allergic type of the diseases that are increasing, c.f. (Nielsen et al., 2002). This increase may have several reasons.

Changes may have occurred in the pattern of exposure of the immune system (Herz et al., 2000) due, for example, to lower numbers of siblings with consequent reduction in sharing of infections and decreased contact with farmyard animals (von Mutius, 2000). Anthropogenically induced chemicals and pollutants may promote sensitisation because of adjuvant effect (Kapsenberg, 1996; Kimber, 1998; McConnell et al., 2002). Adjuvants include, for example, diesel exhaust particles (Peterson and Saxon, 1996; Parnia and Frew, 2001; Løvik et al., 1997; Fujimaki et al., 1994; Nilsen et al., 1997). Also, phthalates as well as their degradation products (monophthalates) may be adjuvants (Oie et al., 1997; Jaakkola et al., 1999; Larsen et al., 2001a,b). That may also apply to surfactants (Clausen et al., 2000; Cashner et al., 1980).

Higher exposure levels of allergens, e.g. from house dust mites, pets, pollen and moulds, may contribute to the increase (Nielsen et al., 2002). Nevertheless, very high levels of childhood exposures to cat allergens have also been associated with development of tolerance cf. (Platts-Mills et al., 2000). Immunological tolerance is a state of non-responsiveness to a specific antigen induced by previous exposure to the same antigen (Lowrey et al., 1998). Respiratory tolerance may involve multiple mechanisms and depend on antigen doses (Lowrey et al., 1998), and may, for example, be caused by suppressor/inhibitory T cells that secrete cytokines (i.a. interleukin-4 (IL-4), IL-10 and transforming growth factor β -1 (TGF- β 1)), or due to suppression through cell–cell contact-dependent processes (Hoyne et al., 2000; Lowrey et al., 1998).

Lymphoid tissue of the respiratory tract may play an important role in sensitisation (Zuercher et al., 2002) and development of tolerance (Kaya et al., 2002). One important obstacle to overcome, is to circumvent the predominantly default response

of tolerance to non-pathogenic soluble protein antigens when administered to the mucosal surfaces (Lowrey et al., 1998; Holt, 1994). The model immunogen, OVA, was used within an exposure range down to the exposure level of 100 $\mu\text{g}/\text{m}^3$, which is at the exposure levels of cow and pig allergen, the $\mu\text{g}/\text{m}^3$ level, and which may cause sensitisation in humans (Heederik et al., 1999). We used $\text{Al}(\text{OH})_3$ as the model adjuvant. First, it induces a strong Th2 response that promotes development of IgE and IgG1 antibodies (Cox and Coulter 1997; Lindblad 1995). Also, IgE production was stimulated after parenteral administration (Clausen et al., 2000; Larsen et al., 2001a). Additionally, $\text{Al}(\text{OH})_3$ has adjuvant effect in relation to exposures of the respiratory mucosa (Freihorst et al., 1988; Sakamoto et al., 1984; Fujimaki et al., 1986), suggesting that $\text{Al}(\text{OH})_3$ should serve as a model adjuvant in relation to airway exposures.

Recently, our group has developed a screening model for hazard identification of adjuvant effects of chemicals that uses subcutaneous administration of ovalbumin (OVA) together with the test substance (Clausen et al., 2000; Larsen et al., 2001b,a). However, a model is needed for risk assessment that uses the natural sensitisation route, airborne exposure. Development of an inhalation model for adjuvant effect is the overall purpose of this study.

2. Materials and methods

2.1. Animals

Inbred BALB/cJ female mice (Bomholtgård Breeding and Research Centre, Denmark), were 6–8 weeks old and weighed 15–20 g at the beginning of the experiments. Drinking water and food (Altromin no. 1324, Christian Pedersen, Ringsted, DK) were available ad lib. Light/dark cycles were 12/12 h. Room temperature (RT) was maintained at 19–21 °C and relative humidity at 40–60%. The mice were housed up to 10 animals per cage. The animals were weighed before each exposure as changes in weight may be used as a general health indicator (Timbrell, 1991). Treat-

ments of the animals followed procedures approved by The Animal Experiments Inspectorate, Denmark.

2.2. Exposure protocols

The animals were exposed to aerosols with different concentrations of OVA (grade V, Sigma Aldrich) alone or combined with the adjuvant aluminium hydroxide ($\text{Al}(\text{OH})_3$, Alhydrogel, Superfos, Denmark). Control animals were exposed to aerosols of phosphate buffered saline (PBS) alone. In one protocol (Fig. 1a), mice were exposed 20 min per day for 10 consecutive days to aerosols generated from solutions with 25 mg/l to 10 g/l OVA alone or in combination with 0.5% $\text{Al}(\text{OH})_3$. Another protocol (Fig. 1c) used aerosols generated from solutions with 1.25 or 25 mg/l OVA alone or in combination with 0.5% $\text{Al}(\text{OH})_3$. Additionally, 10 g/l OVA was used in combination with $\text{Al}(\text{OH})_3$. Animals were exposed for 10 consecutive days followed by once-weekly aerosol exposure for 6 weeks. All aerosol exposures were for 20 min per day. A positive OVA control group and a negative PBS control group were included. The positive OVA control group was first sensitised with OVA in the standardized injection regime (Clausen et al., 2000) and then challenged once per week for 6 weeks (cf. Fig. 1d) with an aerosol generated from a feeding solution of 25 mg/l OVA (The OVA/OVA control group). The negative PBS control group was injected with PBS and challenged with an aerosol of PBS, (the PBS/PBS control group), once-weekly for 6 weeks. In several cases, experiments were repeated and the similar groups were pooled.

2.3. Exposure conditions

The exposure chamber was a vertical stainless steel cylinder (diameter: 40 cm and height: 37 cm) with a hemispherical lid and bottom made of glass. Aerosols were introduced from the top of the upper lid. Ports for exposures of 10 mice were at the same horizontal level. Additionally, the chamber had ports for sampling of aerosols at the breathing zone of the mice. The airflow rate in the chamber was 20 l/min. Each mouse was placed

within a sealed glass tube that allowed head-only exposures after attachment to the exposure chamber. In some experiments, the tubes were used as 'body' plethysmographs, allowing simultaneously determination of i.a. tidal volume, respiratory rate and airflow during in- and expiration (Vijayraghavan et al., 1993, 1994; Boylstein et al., 1996, 1995).

2.4. Aerosol generation

The aerosols were generated by a PARI LC STAR nebuliser (PARI GmbH, Germany) with an air pressure of 2 bar. The feeding solutions used for aerosol generation were 1.25 mg/l (0.000125%), 25 mg/l (0.0025%), 500 mg/l (0.05%), 1 g/l (0.1%) or 10 g/l (1%) OVA, respectively. The percentage is % w/w, which can be considered equal to % w/v as the final solutions have densities of about one. The concentration of $\text{Al}(\text{OH})_3$ in the feeding solutions was 0.5% (w/v). For the sake of brevity, concentrations reported in per cent are given as % without any further indication of the type. The aerosols were diluted with room air and led through the exposure chamber.

2.5. Aerosol size distribution

Particle aerodynamic diameters are major predictors of particle depositions in the respiratory tract (Snipes 1989). The aerodynamic diameter distributions were determined during simulated exposure conditions with aerosols of PBS and 10 g/l OVA in PBS. Aerodynamic diameters (μm) were measured in the breathing zone of the mice using a Marple Personal Cascade Impactor, Model no. 296 with a Model 290-IA In-Line Adaptor (Sierra Instruments, Graseby) and obtained gravimetrically (Rubow et al., 1987). The particles were gathered in mass fractions afterwards: smaller than 0.6 μm , from 0.6–1.6 μm and larger than 1.6 μm (Table 1). The mass fractions for 500 mg/l OVA were assumed to have a mass distribution, which was the mean of the two characterised aerosols. Due to the low OVA content, the mass distribution of OVA aerosols from solutions of 25 and 1.25 mg/l was assumed to be equal to the mass distribution of PBS. It was further assumed that

Sensitisation protocols

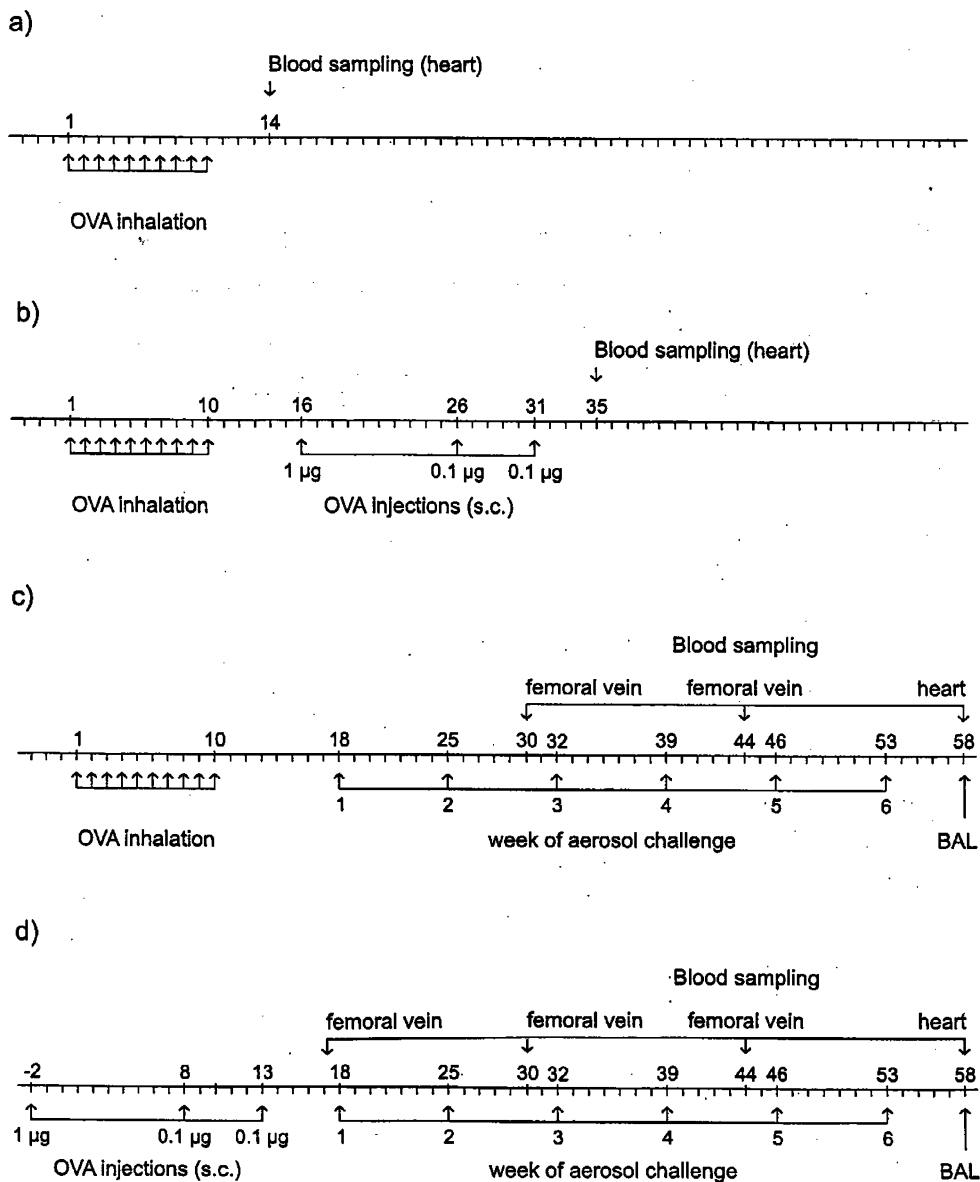


Fig. 1. Schematic drawing of the 4 different sensitisation protocols used. OVA inhalation indicate inhalation of OVA aerosols (1.25, 25, 500 mg/l, 1 or 10 g/l) alone or in combination with 0.5% Al(OH)₃. Each aerosol exposure was for 20 min/day. Days of blood sampling are indicated as well as BAL.

Table 1
Mass fractions (%) of aerosol particles

Aerosol	Diameter (μm)		
	$0 < d < 0.6$	$0.6 \leq d < 1.6$	$1.6 < d$
PBS	54 ^a	33	13
OVA (10 g/l) in PBS	26	51	23

^a Mass fractions in percent.

addition of $\text{Al}(\text{OH})_3$ did not change the mass distributions.

2.6. Concentration in the exposure chamber

The concentration of particles (mg/m^3) in the exposure chamber was determined during all exposures except for the 1 g/l OVA+ $\text{Al}(\text{OH})_3$ group, due to technical difficulties. Samples were collected on Teflon filters (FluoroporeTM Membrane filters, 0.5 μm , Millipore A/S, Denmark) that were placed in a closed-face 25-mm cassette. Filters were placed in line with a motor driven pump (Universal Sample Pump, Model 224-PCXR4, SKS Inc., USA), or laboratory vacuum, and a critical orifice to obtain a presampling flow rate of 1.8 l/min through the filters. The flow rate was also determined after the end of collection, allowing determination of the mean flow rate during the sampling period. Sensitivity for the dust gravimetric analysis was 0.001 mg.

The relation, $C_{\text{total}} = W/(t \times Q)$, was used to calculate the mass concentration of particles in the exposure chamber, where W equals the weight of particles gathered on the Teflon filter, t equals time of exposure and Q equals the airflow through the filter. The total OVA concentration (C_{OVA}) was obtained from the concentration of particles in the exposure chamber (C_{total}) that were corrected for the fractional content of OVA in the feeding solutions, which consisted of the dry matter OVA, PBS, NaCl and $\text{Al}(\text{OH})_3$.

2.7. Estimation of total and regional deposition of aerosols

The inhaled dose was calculated from the product of the respiratory rate (f), tidal volume

(VT), concentration of OVA and length of the exposure period (t). Thus, the inhaled dose = $C_{\text{OVA}} \times f \times VT \times t$. The f value, 245 respirations per min, and the VT value, 0.15 ml, were obtained from a group of four naïve mice. These values are in overall agreement with previously obtained results (Nielsen et al., 1999) and were used for the further calculations. The total length of the exposure period was 200 min (10 times 20 min of exposure) in protocol a and b (Fig. 1) and 320 min (16 times 20 min of exposure) in protocol c (Fig. 1).

The total deposited doses and the doses deposited in the different parts of the respiratory tract and gastrointestinal tract were obtained from the inhaled dose, the distribution of mass aerodynamic diameters of the aerosols and the percentage of the particles deposited in the different mass aerodynamic diameter ranges. Deposition fractions were from (Raabe et al., 1988). As nearly all particles with a diameter from 3.5 μm and above were found in the upper respiratory tract (Raabe et al., 1988) and the gastrointestinal tract, deposition fractions for particles in the range 3.5–9.7 μm were averaged for the present study. Deposition fractions of the monodisperse aerosols (Raabe et al., 1988) with mass aerodynamic diameter 0.3, 1.1 and ≥ 3.5 μm , respectively, were accepted to represent deposition fractions in this study for particles < 0.6 , 0.6–1.6 and ≥ 1.6 μm .

The cumulated deposition of OVA is shown in Table 2. In a pilot study, 1% Evans Blue was added to 1% (10 g/l) OVA in PBS and used for aerosol exposure of mice. Autopsy showed that Evans Blue, and thus presumably OVA, was deposited in the lungs, the upper airways and the gastrointestinal tract (data not shown), which is in qualitative agreement with the estimated deposition patterns.

Our quantitative estimates are reasonable. The deposition in the lungs from a 10 g/l OVA solution for a single 20 min exposure period was 10 μg (calculated from Table 2), which is in reasonable agreement with a measured deposition, 3.35 μg , in a similar experiment with BALB/cAnN mice (Seymour et al., 1998). In another experiment, BALB/c mice were exposed for 5 min to a 1% OVA solution with aerosol particle size, 3–5 μm ,

Table 2

Concentrations of OVA in solution used for aerosol generations, concentrations of OVA in the breathing zone of the mice, inhaled OVA doses and deposited OVA doses

Concentration in solution	Number of exposures ^a	Air concentration during exposure (mg/m ³) ^b	Cumulated doses (μg)			
			Inhaled	Deposited in lungs	Deposited in upper respiratory tract	Deposited in GI
10 g/l OVA	10	54.4	401.8	103.2	55.9	126.4
500 mg/l OVA	10	4.6	33.9	11.0	4.1	8.9
500 mg/l OVA + Al(OH) ₃ ^c	10	3.8	28.2	9.1	3.4	7.4
25 mg/l OVA	10	0.2	1.2	0.5	0.1	0.3
25 mg/l OVA + Al(OH) ₃ ^c	10	0.1	0.9	0.4	0.1	0.2
10 g/l OVA + Al(OH) ₃ ^c	16	32.6	385.4	99.0	53.6	121.2
25 mg/l OVA	16	0.17	2.0	0.8	0.2	0.4
25 mg/l OVA + Al(OH) ₃ ^c	16	0.14	1.6	0.6	0.2	0.3
1.25 mg/l OVA	16	0.005	0.059	0.023	0.006	0.012
1.25 mg/l OVA + Al(OH) ₃ ^c	16	0.005	0.058	0.023	0.006	0.0012

^a Exposures, 20 min/day, were either for 10 consecutive days or for 10 consecutive days followed by 1 weekly exposure for 6 weeks.^b The median coefficient of variance for all experiments was 18.5%.^c The concentration of Al(OH)₃ was 0.5%, cf. [Section 2](#).

(Holt et al., 1981) that is somewhat larger than in our study. Proportional scaling to a single 20 min exposure period suggests a total OVA deposition of 45 µg compared to 29 µg in this study (obtained from Table 2) and a lung deposition of 1 µg compared to 10 µg in our study.

2.8. Test for tolerance

No or low antibody production may be due to several reasons: the OVA dose was too low, the exposure period and the period before collection of blood was too short, and finally tolerance may have developed. These conditions can be distinguished by means of the standardised parenteral OVA scheme, which in itself is known to cause antibody production in naïve animals. Such a procedure will in the first cases cause development of antibodies, whereas no antibodies or decreased antibody levels will appear if tolerance has developed (Lowrey et al., 1998). That is a commonly used procedure (Holt et al., 1981, 1987; Fox and Siraganian 1981; van Halteren et al., 1997; Seymour et al., 1998; Tamura et al., 1994; Tsitoura et al., 2000). We used the following procedure: 6 days after the tenth aerosol exposure animals from protocol b were sensitised subcutaneously (s.c.) with OVA, 1 µg in 100 µl 0.9% saline, followed by two booster injections, 0.1 µg OVA in 100 µl 0.9% saline, the first one 10 days after the first injection, the second 15 days after the first injection (cf. Fig. 1b).

2.9. Blood sampling

Blood was collected (Fig. 1) by heart puncture or animals were bled from the distal part of the lateral femoral vein (about 200 µl blood) as described (Aaberge et al., 1992). For heart puncture, mice were anaesthetized with Hypnorm/Dormicum, for details cf. (Clausen et al., 2000). Serum was prepared and stored at –80 °C until analysed.

Blood samples from the heart were tested for OVA specific IgE and IgG1 antibodies in protocols a and b (Fig. 1). From protocols c and d (Fig. 1), all blood samples from the femoral vein were tested for OVA specific IgE antibodies and, whenever enough serum was available, it was

also tested for IgG1 antibodies. Blood samples from the heart were tested for IgE, IgG1 and IgG2a antibodies.

2.10. Quantification of OVA specific antibodies in serum

OVA specific IgE antibodies in serum were measured by a capture ELISA according to (Ormstad et al., 1998; Larsen et al., 2001b). The standard serum for IgE was produced using the standard injection regime. As the samples are compared to the IgE standard, the amount of IgE is expressed in arbitrary units (a.u.). The standard IgE serum contained 1200 a.u. by comparison with a standard serum, a kind gift from Dr Løvik, National Institute of Public Health, Norway. The Norwegian IgE standard was designated as 100 units. The background level for the assays is about 0.15 absorbance values and the detection limit about 10 units. This ELISA was shown to give results that were in agreement with those obtained by heterologous mouse-rat passive cutaneous anaphylaxis test (Clausen et al., 2000).

The amount of IgG1 was measured by a direct ELISA (Larsen et al., 2001b). The standard serum for IgG1 was produced using the same method as for the IgE standard. As the samples are compared to the internal IgG1 standard, the amount of IgG1 is expressed in a.u. The standard IgG1 serum contained about 30 000 a.u. An absorbance value of 1.000 was defined as 100 a.u. The background level was about 0.10 absorbance values. The limit of detection was about 10 units.

The amount of OVA specific IgG2a was measured by the same method as IgG1 using alkaline phosphatase conjugated goat anti-mouse IgG2a antibodies (Southern Biotechnology Associates Inc.) diluted 1:2000 in Tris/HCl pH 7.4. The internal IgG2a standard used in this assay was produced by sensitising female BALB/cJ mice with OVA (100 µg) in Freund's complete adjuvant and giving two booster injections 14 days apart with OVA (100 µg) in Freund's incomplete adjuvant. The standard IgG2a serum contained 140 000 a.u. An absorbance value of 1.000 was defined as 100 a.u. and the background levels for this assay were

about 0.10 absorbance values. The limit of detection was about 10 a.u.

2.11. Bronchoalveolar lavage

After blood was collected by heart puncture, lung lavage was performed with saline. Thus, 3×1 ml of saline was instilled through a tracheal cannula and the bronchoalveolar lavage (BAL) fluid recovered was pooled (mean: 2.8 ± 0.16 ml) and centrifuged (300 g, 4 °C, 10 min). The supernatant was discarded and the pellet was resuspended in 100 µl PBS buffer containing heparin (20 IE/ml) and serum albumin (0.03%). The total number of cells was determined using a haemocytometer. For differential counts, cytopsin preparations were made (Cytospin[®]2, StatSpin[®] Inc. Massachusetts, USA) (1000 g, 4 min, RT). Slides were stained with May-Grünwald/Giemsa. After blinding, slides were evaluated by one person. Cells were identified by standard morphology and differentiated into neutrophils, eosinophils, epithelial cells, lymphocytes and macrophages. Two hundred cells were counted per slide and the percentage of each cell type was calculated.

2.12. Statistics

Responder rates of two groups were compared by means of Fisher's exact test (Fisher) and responder rates of three and more groups were compared by χ^2 -test. Median levels of two independent groups were compared by means of Mann-Whitney *U*-test (Mann Whitney) and median levels of three and more independent groups by means of Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis). The Wilcoxon signed-rank test was used for comparison of two related samples (Wilcoxon). The non-parametric 1-sample sign test was used to estimate 95% confidence intervals with cells in BAL fluid. *P* values below 0.05 were considered statistically significant. Median value, 25 and 75% percentiles were used for the presentation of results in the figures.

3. Results

3.1. Animal weights

No apparent biological difference was detected between the PBS control group and the OVA and OVA + Al(OH)₃ exposure groups, suggesting that exposures caused no systemic toxic effects (data not shown).

3.2. OVA specific IgE and IgG1 4 days after ten aerosol exposures (protocol a Fig. 1)

Ten consecutive days of OVA aerosol exposures, 25 mg/l–10 g/l, with or without Al(OH)₃ adjuvant (0.5%) did not result in production of OVA specific IgE antibodies 4 days after the last aerosol exposure. Some of the exposure groups produced a minimal amount of OVA-specific IgG1. Thus, the median IgG1 production in the 1g/l OVA + Al(OH)₃, 500 mg/l OVA and 500 mg/l OVA + Al(OH)₃ groups were 80, 13 and 13 a.u., respectively. The median level was zero in the 10 g/l OVA, 25 mg/l OVA and 25 mg/l OVA + Al(OH)₃ groups (data not shown).

3.3. OVA specific IgE production after 10 aerosol exposures followed by s.c. OVA administration (protocol b Fig. 1)

After s.c. administration of OVA, IgE production was observed in 13/15 animals in the PBS aerosol group, whereas 0/10, 4/10 and 5/10 animals responded in the 10 g/l, 500 and 25 mg/l OVA groups, respectively. These responder rates were significantly different (χ^2 -test, $P = 0.005$). The responder rate in the group exposed to 10 g/l OVA was significantly lower than in the PBS control group (Fisher, $P < 0.05$). The median IgE antibody production in the PBS and the OVA groups (Fig. 2) were also significantly different (Kruskal-Wallis, $P < 0.001$). The exclusion of the PBS group from the analysis resulted in a significant difference between the OVA groups themselves (Kruskal-Wallis, $P < 0.05$). Furthermore, the median levels of IgE antibody production in all OVA groups were lower than that of the PBS group (Mann Whitney, $P < 0.05$), which indicated

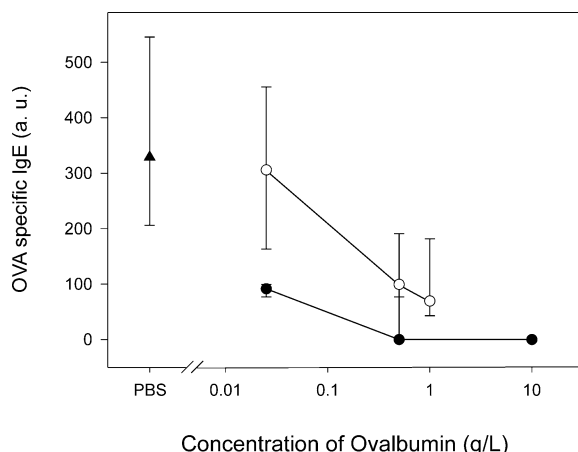


Fig. 2. Median values of OVA specific IgE antibodies in serum after 10 days of consecutive aerosol exposures for 20 min/day with the OVA solutions, 0.025, 0.5 and 10 g/l, respectively, used for aerosol generation (●). The OVA solutions, 0.025, 0.5 and 1 g/l, respectively, were used in combination with $\text{Al}(\text{OH})_3$ (0.5%) (○). Control animals were exposed to aerosols of PBS (▲). All aerosol exposures were followed by the standard injection scheme consisting of three sc injections with OVA (protocol b, Fig. 1), which in itself would induce production of IgE. Blood was collected 4 days after the last injection, day 35 of the experiment. The error bars represents 25 and 75% percentiles.

development of full or partial tolerance with the used OVA aerosols.

After s.c. administration of OVA to the OVA + $\text{Al}(\text{OH})_3$ aerosol groups, the responder rates were 8/10, 8/10 and 9/10 in the groups exposed to 1 g/l, 500 and 25 mg/l of OVA, respectively. The responder rates were not significantly different (χ^2 -test). The median antibody productions in the PBS and OVA + $\text{Al}(\text{OH})_3$ groups were significantly different (Kruskall-Wallis, $P < 0.02$). IgE antibody production in the 1 g/l OVA group was significantly lower (Mann Whitney, $P < 0.01$) than in the PBS group. The lower production in the 500 mg/l OVA group was borderline to significant (Mann Whitney, $P = 0.06$) whereas the median level of the 25 mg/l OVA group (306 a.u.) was at the same level as in the PBS group (329 a.u.) (Fig. 2). This indicated that the $\text{Al}(\text{OH})_3$ adjuvant completely counteracted the development of tolerance with the 25 mg/l OVA solution.

3.4. OVA specific IgG1 production after 10 aerosol exposures followed by s.c. OVA administration (protocol b Fig. 1)

When the 10 consecutive days of OVA aerosol exposures were followed by the standard OVA injection regime, the responder rate in the PBS injection group was 11/15. The responder rates in the OVA exposure groups 25 mg/l, 500 mg/l and 10 g/l were 6/10, 9/10 and 10/10, respectively, and not significantly different (χ^2 -test, $P = 0.4$). Nevertheless, the median IgG1 antibody productions in the PBS and the OVA groups (Fig. 3) were significantly different (Kruskall-Wallis, $P < 0.001$). The exclusion of the PBS group from the analysis revealed a significant difference between the OVA groups themselves (Kruskall-Wallis, $P < 0.01$). The median levels of IgG1 antibody production in the 500 mg/l and 10 g/l OVA groups were higher than that of the PBS group (Mann Whitney, $P < 0.01$).

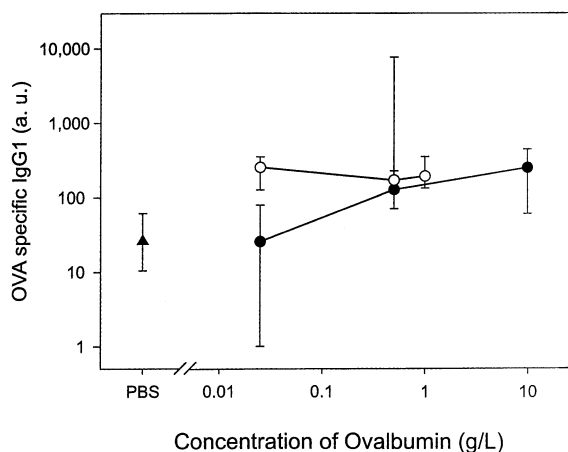


Fig. 3. Median values of OVA specific IgG1 antibodies in serum after 10 days of consecutive aerosol exposures for 20 min/day with the OVA solutions, 0.025, 0.5 and 10 g/l, respectively, used for aerosol generation (●). The OVA solutions, 0.025, 0.5 and 1 g/l, respectively, were used in combination with $\text{Al}(\text{OH})_3$ (0.5%) (○). Control animals were exposed to aerosols of PBS (▲). All aerosol exposures were followed by the standard injection scheme consisting of three sc injections with OVA, which in itself would induce production of IgG1. Blood was collected 4 days after the last injection, day 35 of the experiment. The error bars represents 25 and 75% percentiles.

The median levels in the 25 mg/l OVA group and the PBS group were not statistically different.

The responder rates in each of the three groups exposed to the respective concentrations of OVA+Al(OH)₃ followed by the injection regime were 10/10. The responder rates in the PBS (11/15) and the three OVA+Al(OH)₃ groups (10/10) were not statistically different (Fisher). The median levels of IgG1 production in the PBS and the OVA+Al(OH)₃ groups (Fig. 3) were significantly different (Kruskal-Wallis, $P < 0.001$) whereas the IgG1 levels were not significantly different in the OVA+Al(OH)₃ groups themselves (Kruskal-Wallis, $P > 0.8$). The production in the OVA+Al(OH)₃ groups were all significantly higher than the production in the PBS group (Mann Whitney, $P < 0.01$). At 25 mg/l the median antibody production in the OVA+Al(OH)₃ group was 10 times higher than the corresponding median level in the OVA group, which is highly significant (Mann Whitney, $P = 0.002$). Thus, Al(OH)₃ showed a strong adjuvant effect at this exposure level.

3.5. Extension of the exposure period

Due to minimum of tolerance on IgE, counteraction of tolerance by Al(OH)₃, and adjuvant effect of Al(OH)₃ on the IgG1 response, the 25 mg/l OVA level was used in an extended study with 10 consecutive days of aerosol exposure followed by once-weekly aerosol exposure for 6 weeks. Additionally, a 20 times lower OVA level, 1.25 mg/l and a 10 g/l OVA+Al(OH)₃ group were included.

Parenteral OVA administration followed by aerosol exposures to OVA are commonly used to produce high amounts of OVA specific IgE (Foster et al., 1996; Temelkovski et al., 1998) and IgG1 antibodies (Neuhaus-Steinmetz et al., 2000) as well as pathological conditions with eosinophilic infiltration in the lungs (Neuhaus-Steinmetz et al., 2000; Foster et al., 1996; Temelkovski et al., 1998) that resemble human asthma (Busse and Lemanske., 2001; Foster et al., 1996). We therefore included a similar group (OVA/OVA) in which the 10 consecutive exposures were replaced by the standard injection scheme followed by six once-weekly OVA aerosol expo-

sure for 20 min/day with 25 mg/l OVA (protocol d, Fig. 1). A negative control group with sham exposures with PBS injections and PBS aerosol exposures (PBS/PBS group) was included for comparison of BAL fluid cells.

3.6. OVA specific IgE antibody production during the 16 aerosol exposures (protocol c Fig. 1)

The group exposed to 1.25 mg/l OVA did not produce any IgE antibodies at all, and this is the no-observed-effect level (NOEL) with OVA alone. The group exposed to 1.25 mg/l OVA+Al(OH)₃ produced no antibodies until after the sixth weekly aerosol exposure. Four animals out of ten responded. The median IgE level was zero and the 25 and 75% percentiles were 0 and 80, respectively. Thus, the Al(OH)₃ adjuvant was able to lift the IgE response from the NOEL (1.25 mg/l OVA alone) to a response level that corresponded to the

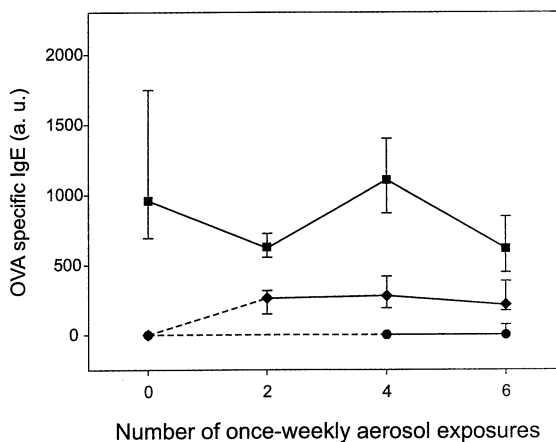


Fig. 4. Median values of OVA specific IgE antibodies after 10 consecutive days of aerosol exposure followed by 1 weekly aerosol exposure for up till 6 weeks. Exposures were for 20 min/day. Mice were exposed to aerosols of 25 mg/l OVA alone (●) or in combination with Al(OH)₃ (◆). Note: the 0 week values are from protocol a, Fig. 1. A positive control group (protocol d, Fig. 1) was included that was sensitised by the standard injection regime, consisting of three s.c. injections with OVA, and afterwards exposed once weekly for 6 weeks to an aerosol from a 25 mg/l OVA solution (■). The error bars represents 25 and 75% percentiles.

20 times higher OVA level of the 25 mg/l OVA group, c.f. below.

The group exposed to 25 mg/l OVA did not produce IgE antibodies until after the sixth once-weekly aerosol exposure. Four animals out of nine responded, i.e. the median IgE level was zero (Fig. 4) with 25 and 75% percentiles at 0 and 98, respectively. Aerosol exposure for 10 consecutive days followed by 2 weekly aerosol exposures induced the production of OVA specific IgE (median 262 a.u.) in the animals exposed to 25 mg/l OVA + Al(OH)₃. The IgE level was maintained throughout the remaining part of the exposure period (Fig. 4) and thus, no booster effect was apparent with the remaining exposures. All animals in the 25 mg/l OVA + Al(OH)₃ group responded during the once-weekly exposures (responder rate after 6 weekly exposures: 21/21) and the group had a significantly higher IgE production after 6 weekly exposures than that of the 25 mg/l OVA group (Mann Whitney, $P = 0.001$). However, the IgE level in the 25 mg/l OVA + Al(OH)₃ group did not reach the IgE level of the OVA/OVA group (Mann Whitney, $P = 0.002$), cf. below.

No IgE was produced in the 10 g/l OVA + Al(OH)₃ group until after the sixth weekly aerosol exposure. After six once-weekly exposures, very little IgE (median 76) was produced, but all animals responded.

Subcutaneous sensitisation with OVA in the OVA/OVA control group resulted in a high production of IgE (Fig. 4) 4 days after the injection scheme. All ten animals responded during the 6-week period. The high IgE production was maintained during the entire period of weekly aerosol exposures with the 25 mg/l OVA solution.

3.7. OVA specific IgG1 antibody production during the 16 aerosol exposures (protocol c Fig. 1)

Ten consecutive OVA exposures, followed by once-weekly exposure for 6 weeks, resulted in a low OVA specific IgG1 production in the 1.25 mg/l (i.e. responder rate 2/10). However, none of the ten animals in the 1.25 mg/l OVA + Al(OH)₃ group responded. No antibody production occurred in the 25 mg/l OVA group (responder rate 0/14).

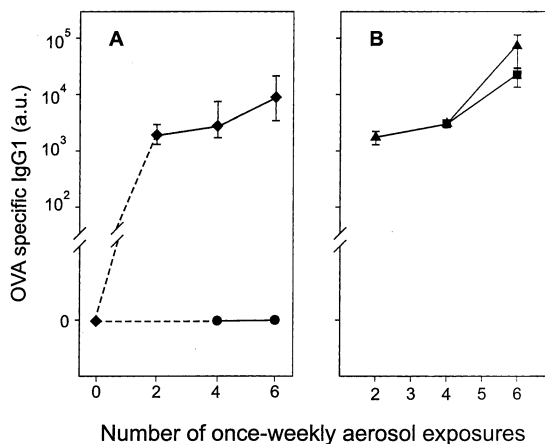


Fig. 5. Median values of OVA specific IgG1 antibodies after 10 consecutive days of aerosol exposure or the standard injection scheme followed by 1 weekly aerosol exposure with the same aerosol for up till 6 weeks. Results from the 6 weekly exposures are indicated. Exposures were for 20 min/day. (A) Mice were exposed to aerosols of 25 mg/l OVA (●), 25 mg/l OVA + Al(OH)₃ (◆). Note: the 0 week values are from protocol a, Fig. 1. (B) Mice were exposed to aerosol of 10 g/l OVA + Al(OH)₃ (▲) or the standard OVA injection scheme (protocol d, Fig. 1), consisting of three s.c. injections with OVA, followed by OVA (25 mg/l) aerosol exposure for 6 weeks (■). The error bars represents 25 and 75% percentiles.

When ten aerosol exposures in the 25 mg/l OVA + Al(OH)₃ group was followed by two additional weekly aerosol exposures, a high IgG1 production (median 1856 a.u.) was observed in the four animals where enough serum was available for the ELISA method (Fig. 5A). The responder rate after the sixth weekly exposure was 21/21 and the median IgG1 production increased to 9053 a.u. (Fig. 5A). The increase from the fourth to the sixth week was statistically significant (Wilcoxon, $P < 0.01$) when comparing 15 pairs of data from animals available from both periods. The responder rate after the sixth weekly exposure was significantly higher than in the 25 mg/l OVA group (Fisher, $P < 0.001$) and the median IgG1 production was high compared to the lack of IgG1 production in this 25 mg/l OVA group. This OVA level showed a significant adjuvant effect of Al(OH)₃ and a booster effect of OVA exposure.

After 10 days of aerosol exposure and two once-weekly aerosol exposures, the IgG1 antibody

production in the 10 g/l OVA+Al(OH)₃ group reached a median value of 1763 a.u. (Fig. 5B), based on two serum samples where enough serum was available. After 4 weekly exposures, serum was available from 9 animals that all responded with a higher IgG1 production. Thus, the median value exceeded 3000 a.u. After 6 weekly aerosol exposures, 10 of 10 animals responded and the IgG1 production had increased significantly from the 4 weeks of exposure to the median value 77 343 (Wilcoxon, $P < 0.01$). This value is 8.5 times higher than the production in the 25 mg/l OVA+Al(OH)₃ group, and borderline to statistically significant (Mann Whitney, $P = 0.052$). The repeated OVA exposures showed a booster effect at the 10 g/l OVA level.

After 4 weekly exposures, serum samples were available from three animals in the OVA/OVA group. The group median IgG1 level was 3000 a.u. After 6 weekly exposures, 10/10 animals responded and a high IgG1 level was apparent, median 22 811 a.u. This level was 2.5 times higher than the production in the 25 mg/l OVA+Al(OH)₃ group, but not significantly different (Mann Whitney).

3.8. OVA specific IgG2a production

The production of IgG2a antibodies was only studied after 6 weekly exposures, due to the limited amount of serum that was available from femoral vein puncture. The group exposed to 10 g/l OVA+0.5% Al(OH)₃ produced a median level of 176 a.u. and 7/9 animals responded (data not shown). In all other exposed groups, the median IgG2a production was zero. However, a minority of the animals responded with a IgG2a production near the limit of detection.

3.9. Bronchoalveolar lavage

Leukocyte, mainly eosinophil, infiltration of the airways is one characteristic feature of human asthma (Djukanovic et al., 1990). As epithelial cell shedding is also another characteristic feature of human asthma (Djukanovic et al., 1990), the lial cells included in the characterisation of BAL cells in addition to the leukocytes. BAL was performed after the end of the sixth weekly aerosol exposure.

The percentage distribution of neutrophils, epithelial cells, lymphocytes, alveolar macrophages and eosinophils in the BAL fluid is shown in Table 3 together with 95% confidence limits. In all groups, epithelial shedding (comprising from 0.3 to 5.5% of the cells) and neutrophil infiltration (comprising from 0.5 to 3% of the cells) were low, demonstrating the absence of any overt respiratory inflammation associated with the exposures to the OVA concentrations in the aerosols. Nevertheless, the eosinophil infiltration in the group that received OVA parenterally followed by aerosol exposures was markedly increased. No meaningful relation was obtained from the absolute cell numbers, probably explained by the low degree or absence of inflammation.

4. Discussion

4.1. Production of OVA specific IgE and IgG1 antibodies after ten consecutive aerosol exposures

We observed no IgE and minimal IgG1 antibody production with protocol a (Fig. 1). Similarly, 12 once-weekly exposures for 6 h to aerosols from 1 mg/l to 1 g/l OVA solutions failed to produce OVA specific IgE antibodies in six inbred strains of mice, whereas low IgG1 antibody levels were detected after five aerosol exposures (Fox and Siraganian, 1981). In another study, BALB/c mice were exposed eight times, once a week for 5 min, to an aerosol from a 1% (10 g/l) OVA solution, eliciting a transient IgE response, which declined by the seventh exposure (Holt et al., 1981). These findings are supported in studies with i.n. instillation of OVA (Wolvers et al., 1997; Tamura et al., 1994) and are in overall agreement with the results from this study. Nevertheless, there are also several studies, using the exposure scheme similar to our protocol a, in which OVA specific IgE antibodies were produced by female BALB/c mice following 10 days of exposures for 20 min/day with a 1% w/v OVA solution (Renz et al., 1992; Larsen et al., 1994; Lack et al., 1994; Renz et al., 1994) but not in C57BL/6 mice (Makela et al., 2000).

Table 3

The percentage distribution of cells in BAL fluid from BALB/cJ mice

Solutions used for generation of aerosol	Neutrophils	Epithelial cells	Lymphocytes	Alveolar macrophages	Eosinophils
PBS injection + PBS aerosol	2.3 (0.5–13)	0.3 (0.0–4.8)	26 (2.9–43)	65 (45–94)	0.0 (0.0–2.8)
1.25 mg/l OVA	3.0 (1.5–7.3)	1.5 (0.3–9.2)	15 (8.3–37)	73 (52–86)	0.0 (0.0–0.5)
1.25 mg/l OVA + Al(OH) ₃ ^a	1.8 (1.2–4.5)	1.8 (0.3–5.8)	14 (3.7–37)	79 (55–92)	0.0 (0.0–1.0)
25 mg/l OVA	2.0 (0.5–5.0)	4.5 (2.3–18)	17 (3.0–39)	59 (48–74)	0.0 (0.0–0.6)
25 mg/l OVA + Al(OH) ₃ ^a	0.5 (0.0–1.0)	5.5 (3.8–11)	24 (6.4–35)	68 (60–77)	0.0 (0.0–0.1)
10 g/l OVA + Al(OH) ₃ ^a	1.0 (0.2–2.4)	1.0 (0.1–2.9)	19 (3.9–24)	79 (75–92)	0.0 (0.0–0.5)
OVA injection + OVA aerosol ^b	2.0 (0.3–5.5)	0.8 (0.0–3.0)	36 (34–43)	53 (46–61)	4.5 (2.3–7.0)

The animals were exposed to aerosols for 10 consecutive days followed by once-weekly aerosol exposure for 6 weeks, except for the injection groups which were sensitised s.c. and boosted twice either with OVA or PBS and then exposed to OVA or PBS aerosols once weekly for 6 weeks. The lung lavage was performed 5 days after the sixth weekly exposure. The numbers represents median values. Values in parenthesis indicates 95% confidence limits.

^a The concentration of Al(OH)₃ was 0.5%, cf. Section 2.

^b OVA injections followed the standard injection regime and aerosols were made from a 25 mg/l OVA solution.

4.2. Ten OVA aerosol exposures caused IgE tolerance

All aerosol exposures for 10 consecutive days resulted in development of tolerance of the IgE formation (Fig. 2), which decreased with decreasing OVA exposure levels. Animals exposed to 10 g/l (1%) OVA were fully tolerant. A limited amount of IgE was formed with 25 mg/l, less than 20% of the level of the PBS group. Similarly, in a study with BALB/cAnN mice, animals were exposed for 20 min/day for 10 consecutive days. The OVA solutions from 10 mg/l to 10 g/l caused concentration-dependent tolerance of the IgE production (Seymour et al., 1998). Indeed, in several studies (Fox and Siraganian, 1981; Holt et al., 1981, 1987) development of tolerance has been reported to occur with OVA aerosols in mice. Also our study showed a 'default' setting to tolerance over a broad exposure range of OVA concentrations.

Tolerance can be induced by antigen administration to the respiratory tract (Hoyne et al., 2000; Lowrey et al., 1998; Holt, 1994) as well as by administration to the gastrointestinal tract (Mayer, 2000; Strober et al., 1998). Both areas are exposed in the present study (Table 2). A single oral dose of 1000 µg OVA was able to induce partial tolerance in BDF1 mice (Saklayen et al., 1984). In another study (Ngan and Kind 1978),

feeding of OVA to SWR mice in drinking water on alternate days for 2 weeks caused tolerance of the IgE response at 100 µg/day, i.e. cumulated dose ≈ 700 µg, whereas no apparent tolerance appeared with 10 µg/day, i.e. cumulated dose ≈ 70 µg. The orally tolerogenic doses are far above the cumulated doses estimated to be deposited in the gastrointestinal tract for all exposure groups with concentrations below 10 g/l OVA (Table 2), suggesting that the tolerance observed at the lower doses is due to effects in the respiratory tract itself. A similar result was obtained previously (Fox and Siraganian, 1981). Our study showed that the degree of tolerance depended on the antigen dose, as has previously been discussed (Lowrey et al., 1998; Seymour et al., 1998).

In a recent study in mice, OVA induced respiratory tolerance through effects from the superficial cervical and internal jugular lymph nodes that drain the nasal mucosa (Wolvers et al., 1999). Similarly in rats, suppressor T cells appeared first in the superficial cervical lymph nodes (McMenamin et al., 1991). It is therefore tempting to speculate that the deposition pattern determined by particle size may influence the development of tolerance versus sensitisation. This is supported by results from studies in which the relative potencies of proteins were studied by intratracheal (i.t.) (Kawabata et al., 1996) and intranasal (i.n.) administration (Robinson et al.,

1996). I.n. administration only resulted in a negligible production of specific IgE antibodies in contrast to a significant IgE response following the i.t. administration. The importance of the deposition patterns may also offer one possible explanation why the same OVA solution may cause either tolerance or sensitisation of the IgE response as different aerosol generators may produce particles of different sizes (Reisner et al., 2001).

4.3. Ten OVA aerosol exposures promoted OVA specific IgG1 antibody production when followed by parenteral OVA

OVA aerosols for 10 consecutive days increased OVA specific IgG1 production in a concentration-dependent manner, when followed by the subsequent administration of parenteral OVA (Fig. 3). The lowest OVA concentration, 25 mg/l, did not increase IgG1 antibody production whereas the higher concentration, 10 g/l, caused maximal IgG1 antibody production. The results suggest that either the OVA aerosol exposures alone were insufficient or the period between exposure and collection of serum was too short to allow for the development of IgG1 antibodies 4 days after 10 aerosol exposures. This latter possibility is probably the cause. Thus, Holt et al. (1981) demonstrated IgG production in a study with aerosol exposure from a 1% (10 g/l) OVA solution for 5 min once a week for 8 weeks followed by subsequent administration of a parenteral sensitisation scheme. Similar to our study, ten consecutive exposures to OVA aerosol for 20 min/day caused a concentration-dependant increase in IgG1 production after i.p. administrated OVA + Al(OH)₃ in BALB/cAnN mice (Seymour et al., 1998). In general, repeated exposure of mice to aerosols with allergens either evoked normal or enhanced IgG production after parenteral sensitisation (Holt and McMenamin, 1989).

4.4. Sixteen OVA aerosol exposures caused dose-dependent OVA specific antibody production

The 16-day exposure scheme showed several results similar to those seen in human allergic

airway diseases. A dose-dependent production of OVA specific IgE was apparent using this scheme with the OVA solutions 1.25 and 25 mg/l, respectively. This is in agreement with experiences from humans, where sensitisation occur in a dose-dependent manner in the low dose range (Nielsen et al., 2002; Platts-Mills, 2001). However, the dose-dependent sensitization in humans may reach an optimum level and after that, higher doses in fact may decrease the sensitization (Platts-Mills, 2001), and, overall, give rise to an inverted U-shaped relationship (Custovic et al., 2001), as is the case for cat allergens (Platts-Mills, 2001; Custovic et al., 2001). As we observed maximum tolerance at the higher concentration of OVA in the 10-day exposure experiments, this suggests a similar type of response with OVA in BALB/c mice. The IgG1 production occurred in a dose-dependent manner.

The Al(OH)₃ adjuvant was able to raise IgE production in the 1.25 and 25 mg/l OVA groups, demonstrating a direct adjuvant effect on IgE production. Also, in agreement with the high degree of tolerance with 10 g/l OVA, IgE production was low although Al(OH)₃ was present during all 16 exposures. The adjuvant effect of Al(OH)₃ was even more pronounced in IgG1 production (Fig. 5A). Overall, we were able to demonstrate a direct adjuvant effect of airborne Al(OH)₃ on the production of the two Th2 cytokine dependent antibodies, IgE and IgG1. This is especially important, as IgE antibodies play a central role in the development of allergic asthma and allergic rhinitis (Platts-Mills, 2001; Busse et al., 2001).

4.5. Inflammatory cells in BAL fluid

None of the protocols (Table 3) using airborne exposures exclusively resulted in an increase in eosinophils in BAL fluid. In contrast to this, parenteral sensitisation, followed by six once-weekly aerosol exposures to a 25 mg/l OVA solution, resulted in a high OVA specific IgE production (Fig. 4) and a conspicuous increase in eosinophils in BAL fluid (Table 3). The BAL fluid only contained a low number of neutrophil and epithelial cells (Table 3). Similar results have been reported in studies with a somewhat higher OVA concentration in the aerosols. Thus, parenterally

induced OVA specific IgE production that was followed by eight OVA aerosol exposures (each on a consecutive day), to a 2 g/l (0.2%) OVA solution for five min/day, increased eosinophils in BAL fluid (De Bie et al., 1998; Hofstra et al., 1998), whereas neither mononuclear cells and neutrophils (De Bie et al., 1998; Hofstra et al., 1998) nor the total cell count increased (Hofstra et al., 1998). In contrast, BALB/c mice sensitised parenterally with OVA and challenged for 20 min twice per week during 4 weeks with an aerosol from a 1% OVA solution experienced a conspicuous increase in lymphocytes, neutrophils and monocytes in BAL fluid, in addition to eosinophils (Neuhaus-Steinmetz et al., 2000). This suggests that low allergen concentration in the aerosols mainly recruited eosinophils whereas high concentrations recruited both eosinophils and other types of inflammatory cells. Virtually no eosinophils were observed in BAL fluid in parenterally sensitised animals exposed to saline aerosols instead of OVA aerosols (De Bie et al., 1998; Hofstra et al., 1998).

4.6. Role of IgG1 antibodies

In mice, both IgE and IgG1 can bind to and cause degranulation of mast cells and basophils (Tumas et al., 2001). In epidemiological studies, the risk factor for asthma is IgE and not IgG antibodies (Platts-Mills, 2001). Also in humans, the presence of IgG specific to cat or dust mite allergen is not significantly associated with asthma risk (Platts-Mills, 2001). The OVA specific IgG1 levels were approximately at the same levels for the 25 mg/l OVA+Al(OH)₃, 10 g/l OVA+Al(OH)₃ and the parenterally sensitised groups (Fig. 5A and B). This demonstrates that the OVA specific IgG1 antibodies cannot account for the eosinophil infiltration in the parenterally sensitised group. The lack of IgG1 mediated effect, under our exposure conditions, may be an advantage of a test for adjuvant effect, as a 'modified' Th2 response in humans involving IgG and IgG4 but without IgE should be regarded as a form of immunologic tolerance (Platts-Mills, 2001).

5. Conclusion

Ten aerosol exposures with OVA solutions with 25 mg/l–10 g/l showed that BALB/cJ mice developed concentration-dependent tolerance on OVA specific IgE production. At higher concentrations, the development of tolerance may be the dominating effect. The minimal tolerance was induced with 25 mg/l OVA. The Al(OH)₃ adjuvant counteracted development of tolerance maximally with the 25 mg/l solution. Extending the exposure period with once-weekly aerosol exposures for 6 additional weeks suggested that the IgE production followed an inverted U-shaped curve where IgE increased from the NOEL (~1.25 mg/l) to 25 mg/l OVA. Both IgE and IgG1 productions were markedly increased by the Al(OH)₃ adjuvant. Eosinophil infiltration, studied from BAL fluid cell content, was correlated with the OVA specific IgE level. Overall, an animal model intending risk assessment of adjuvant effect of asthma in humans should not only take the development of IgE antibodies into account, but also eosinophilic inflammation in the lungs and airway hyperresponsiveness. Overall, our results suggest that detection of adjuvant effects is optimal with low allergen doses, which also may be the most relevant in relation to risk assessment of exposures to the general human population.

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