

Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen

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SUMMARY

Based on the fact that type I allergies are frequently elicited by inhalant allergens, we have established a model of aerosol inhalation leading to allergic sensitization in BALB/c mice. Using this model we studied the effects of aluminium hydroxide ($\text{Al}(\text{OH})_3$), known to enhance IgE antibody responses, compared with cholera toxin (CT), a potent mucosal adjuvant, on the immune response to birch pollen (BP) and its major allergen Bet v 1. Two groups of BALB/c mice were either systemically immunized with recombinant Bet v 1 in $\text{Al}(\text{OH})_3$ and subsequently aerosol exposed to BP allergen, or aerosolized with BP and CT. IgE-mediated skin reactions were only elicited in the mice which had received Bet v1/ $\text{Al}(\text{OH})_3$. Allergen-specific serum IgE and IgG1 antibodies dominated in the $\text{Al}(\text{OH})_3$ group, IgG2a antibody levels to BP and rBet v 1 were markedly higher in the sera of mice exposed to CT with the allergen. IgA antibodies were only detected in the bronchial lavage of the CT-treated group. Moreover, the latter group displayed consistently higher T cell proliferative responses to BP and interferon-gamma production *in vitro*. Thus, the systemic immunization with rBet v 1 in $\text{Al}(\text{OH})_3$ before inhalation of the BP extract promoted a Th2-like immune response, while CT mixed with the aerosolized BP extract rather induced a Th1-like immune response. In an attempt to reverse these ongoing immune responses we could achieve a shift towards a Th0 response. Immunization with BP extract without adjuvant treatment led to undetectable antibody or cellular immune responses. We conclude from the present study that the induction of an immune response to BP allergen after aerosol inhalation can be directed towards a Th1- or a Th2-like response. Once established, the immune response can be modulated.

Keywords allergy aerosol mucosal adjuvant Th1 Th2 BALB/c

INTRODUCTION

In recent years a constant increase in the prevalence of type I (IgE-mediated) allergy has been observed [1]. In areas of the temperate climate zone most allergic reactions are caused by seasonal airborne allergens. Among these, birch pollen represents an important source of allergens. The major allergen of birch pollen is Bet v 1, a 17-kD protein [2]. Immune responses to Bet v1 in allergic and non-allergic individuals have been extensively studied [3–5]. In experimental studies the immune responses to Bet v1 were characterized in different mouse strains, identifying BALB/c mice as a high IgE responder strain [6].

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T helper cells and their products play a central role in the pathogenesis of type I allergy. IL-4, a cytokine produced by Th2 cells, induces proliferation and IgE isotype switching of B cells, whereas interferon-gamma ($\text{IFN-}\gamma$), a Th1 cytokine, inhibits the production and action of IL-4 [7]. In allergic individuals an imbalance in the cytokine regulation, characterized by an overproduction of IL-4 concomitant with reduced $\text{IFN-}\gamma$ secretion, is responsible for the predominant Th2, and thus high IgE, response to allergenic molecules [8–10]. Therefore, therapeutic strategies should aim to modulate the allergic immune responses on the level of T helper cells. In this respect several approaches have been undertaken. Successful specific immunotherapy (SIT), i.e. injecting increasing doses of specific allergen to induce tolerance, has been associated with a shift from the Th2- to Th0/Th1-like immune responses (increased $\text{IFN-}\gamma$ and decreased IL-4 production) in

allergen-specific T cells from allergic patients [11–14]. Recent *in vivo* and *in vitro* studies have shown that also immunogenic peptides, containing T cell epitopes, can act as tolerogens. The fact that reduction of modulation of T cell reactivities can be achieved without the risk of cross-linking IgE antibodies on mast cells has suggested T cell-targeted treatment as a safer method of SIT therapy [15,16].

Another possibility to influence the immune response to an antigen is the use of particular adjuvants. From the experimental studies it is known that parenteral administration of antigen in conjunction with aluminium hydroxide (Al(OH)₃) elicits specific IgE synthesis [17], associated with Th2-like immune responses [7]. In contrast, certain bacterial compounds, such as Freund's complete adjuvant (FCA) [18] or bacterial surface layer (S-layer) [19], can be used as adjuvants for induction of Th1-like immune responses.

The dichotomy of T helper cells can also be influenced by certain mucosal adjuvants. Among these cholera toxin (CT), an exotoxin produced by *Vibrio cholerae*, has been frequently used because of its capacity to enhance the immunogenicity of poor mucosal immunogens [20,21]. It has been reported that oral and/or nasal immunizations of protein antigens with CT induces strong mucosal IgA and serum IgG and IgE responses [22–24]. *In vitro* studies have shown that CT stimulates macrophages to produce IL-1, a cytokine required as a costimulatory signal for Th cells [25]. Analysis of cytokine-specific mRNA suggested that CT acts as adjuvant through selective induction of Th2-type cytokines, at least when administered by the oral route [26]. However, there is evidence that Th1 cytokines are also detectable in restimulation assays [27,28] and some viral antigens administered with CT preferentially enhance Th1-like responses [29].

In the present study we have established a model of aerosol inhalation, leading to sensitization in mice. Thereby we have studied the effects of Al(OH)₃ compared with CT on the immune response to birch pollen and its major allergen Bet v 1. We show that, in contrast to aluminium hydroxide, CT promotes the induction of Th1 responses to inhaled birch pollen allergen as well as modulates an ongoing allergic immune response.

MATERIALS AND METHODS

Animals

Female, 7-week-old BALB/c mice were obtained from Charles River (Sulzfeld, Germany).

Antigens and immunizations

Recombinant Bet v 1 (rBet v 1) was obtained from Biomay GesmbH (Linz, Austria).

Birch pollen (Allergon AB, Engelholm, Sweden) was used for the preparation of a birch pollen extract (BP) according to a modified protocol of [30]. Birch pollen (50 g) was extracted in 500 ml PBS by overnight stirring at 4°C. After centrifugation at 4000 g for 60 min at 4°C the supernatant was filtered and subsequently dialysed (Spectra/Por1; mol. wt 6–8000; Spectrum, Houston, TX) against PBS for 24 h. The protein concentration of the dialysate was determined according to the method of Bradford [31]. The extract was lyophilized and stored at –20°C.

For systemic immunization 1 µg rBet v 1 was mixed with 50 µl PBS and 100 µl Al(OH)₃ (Serva, Heidelberg, Germany; 2 mg/animal) and injected intraperitoneally in a volume of 150 µl.

Aerosol immunization [32] was daily performed with 4 mg BP

extract (corresponding to 1 mg Bet v 1) solution with or without CT (5 µg/aerosol; Sigma, St Louis, MO) during a period of 10 days and another 10 days after a 2-week interval. The allergen suspension was aerosolized through a nebulizer (DeVilbiss nebulizer 646; Somerset, PA) into a chamber with the dimensions of 23.5 × 23 × 20.5 cm. The mice were exposed to 8 ml suspension for 20 min/day (flow 7 l/min at a pressure of 1.5 bar).

Experimental design

Group 1 (*n* = 6) was immunized twice intraperitoneally with rBet v 1 in Al(OH)₃ at days 0 and 14. From day 28 to 38 and day 52 to 62 the mice were daily aerosolized with BP extract.

Group 2 (*n* = 6) was not systemically preimmunized but aerosolized with 4 mg BP extract and CT for 10 days (day 28–38) and after 2 weeks for another 10 days (day 52–62).

Group 3 (*n* = 6) was immunized twice intraperitoneally with rBet v 1 in Al(OH)₃ at days 0 and 14 and subsequently aerosolized with the allergen mixed with CT as described above.

Group 4 (*n* = 6) was aerosolized with 4 mg BP extract mixed with CT on days 0–10 and 24–34 and thereafter immunized twice (days 48 and 62) with rBet v 1 in Al(OH)₃.

Controls were either aerosolized with BP without adjuvants or with PBS. The experimental design of groups 1–4 is summarized in Fig. 1.

Type I hypersensitivity reaction (skin test)

Seven days after the last immunization intradermal skin tests were performed. One hundred microlitres of 0.5% Evans blue (Sigma) were injected intravenously into the tail vein of the mice. Subsequently 30 µl of BP (2.5 µg/ml) and rBet v 1 (2.5 µg/ml) were injected intradermally into the shaved abdominal skin. The mast cell degranulation compound 48/80 (25 µg/ml; Sigma) was used as a positive control and PBS was injected as a negative control. After 15–20 min the mice were killed, skinned and the diameter was measured at the inside of the skin and the area calculated. The colour intensity of the reaction was scored from 0 to 3 according to the individual positive control.

Sampling

Blood samples were taken before and 6 days after the last immunization by tail bleeding, serum prepared and stored at –20°C until analysis.

Bronchoalveolar lavage (BAL) was collected by lavaging the lungs twice with 1 ml of PBS inserted through a small incision in the trachea of the sacrificed mice. Approximately 65% of the initial lavage solution was recovered routinely free from erythrocytes.

Determination of allergen-specific antibody levels in serum and BAL

Microtitre plates (Nunc, Roskilde, Denmark) were coated with BP extract, 50 µg/ml carbonate buffer pH 9.6 and rBet v 1 (2 µg/ml) overnight at 4°C. After washing with PBS–Tween (PBS–T) the plates were blocked with 1% bovine serum albumin (BSA)–PBS–T for 2 h at room temperature. Thereafter serum samples, diluted 1:2000 for IgG1, 1:1000 for IgG2a and 1:20 for IgE antibodies, and undiluted BAL samples were applied in 100-µl volumes and incubated overnight at 4°C. After five washings, anti-mouse IgG1, IgG2a, IgE and IgA antibodies (1:1000; Pharmingen, San Diego, CA) were applied for 4 h at room temperature. Thereafter peroxidase-conjugated mouse anti-rat IgG antibodies (1:1000; Jackson Immuno Labs Inc., West Grove, PA) were applied for

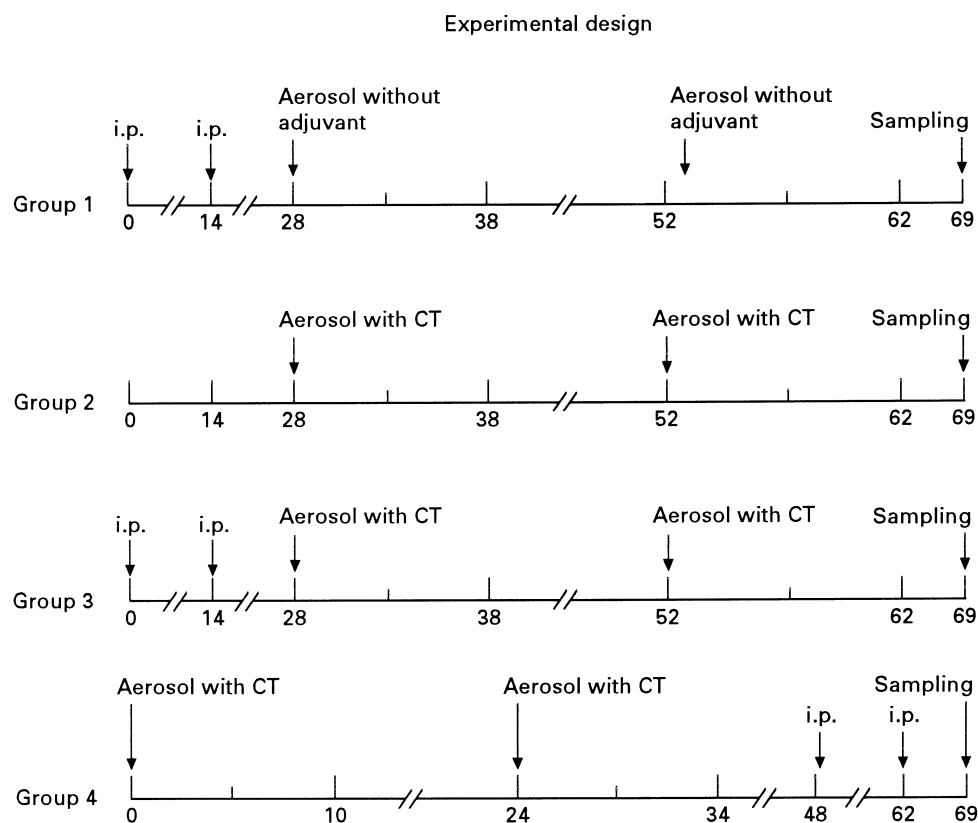


Fig. 1. Immunization protocol of the four experimental groups of BALB/c mice ($n=6$). Protocols of mice ($n=6$) aerosolized with birch pollen (BP) extract alone or with PBS are not shown. i.p., intraperitoneally immunized with rBet v1/Al(OH)₃; CT, cholera toxin.

1 h (30 min 37°C, 30 min 4°C). Colour development was performed with ABTS substrate (1 mg/ml azinoethylbenzthiozoline sulfonic acid substrate; Sigma; and 1 µg/ml H₂O₂) and the absorbance measured at 405 nm with a Dynatech microplate reader. The antibody levels were expressed in optical density (OD) units.

Proliferation assay

At sacrifice, 7 days after the last immunization, spleens were taken aseptically, minced and filtered through sterile steel filters. The erythrocytes were lysed, the cells washed and resuspended in complete medium (RPMI, 10% fetal calf serum (FCS), 0.1 mg/ml gentamycin, 2 mM glutamine, 50 µM 2-mercaptoethanol). The cell suspensions were plated into 96-well round-bottomed plates (Nunc) at a concentration of 2×10^5 /well and stimulated with and without concanavalin A (Con A; 0.5 µg/well; Sigma), BP (2 µg/well) and rBet v 1 (1 µg/well) for 4 days. Thereafter the cultures were pulsed with 0.5 µCi/well ³H-thymidine (Amersham, Aylesbury, UK) for 16 h, harvested and the proliferative response measured by scintillation counting.

Measurement of cytokine production

For determination of IL-4 and IFN-γ production the spleen cell suspensions were cultured with and without Con A (2.5 µg/well) and BP (10 µg/well) in 48-well plates (Costar, Cambridge, MA) at a concentration of 10^7 cells/well. After 24 h and 48 h, supernatants were taken and stored at -20°C until analysis.

The levels of IFN-γ and IL-4 were measured with mouse ELISA kits (Endogene, Cambridge, MA). The sensitivity of the IFN-γ ELISA was <15 pg/ml and the IL-4 ELISA kit <5 pg/ml.

Statistical analysis

For statistical analysis Student's *t*-test and the Mann-Whitney *U*-test were used.

RESULTS

Antibody responses to BP and rBet v 1 induced by immunization with Al(OH)₃ versus CT (group 1, group 2)

Serum IgG1 and IgE antibody levels against BP were higher in the group which was systemically preimmunized with rBet v 1/Al(OH)₃ (group 1) than in the group treated with CT/BP in the aerosol (group 2), even though the difference was not statistically significant (Fig. 2a). In contrast, serum IgG2a antibodies to BP were significantly higher in the CT-treated group than in the Al(OH)₃-preimmunized group. Additionally, IgA anti-BP antibodies were only detected in the BAL of the CT-treated mice.

Analysis of antibody levels to the recombinant Bet v 1 revealed a similar pattern. Whereas serum IgG2a as well as IgA antibodies in BAL were consistently higher in the CT-treated group, IgG1 and IgE anti-rBet antibody levels tended to be higher in the systemically treated group (Fig. 2b).

Skin tests (group 1, 2)

In accordance with the production of allergen-specific IgE antibodies, all mice from the Al(OH)₃-preimmunized group displayed strong immediate-type skin reactions to BP and rBet v 1. In contrast, the CT-treated mice (group 2), which developed preferentially high IgG2a antibody levels to the allergen, showed

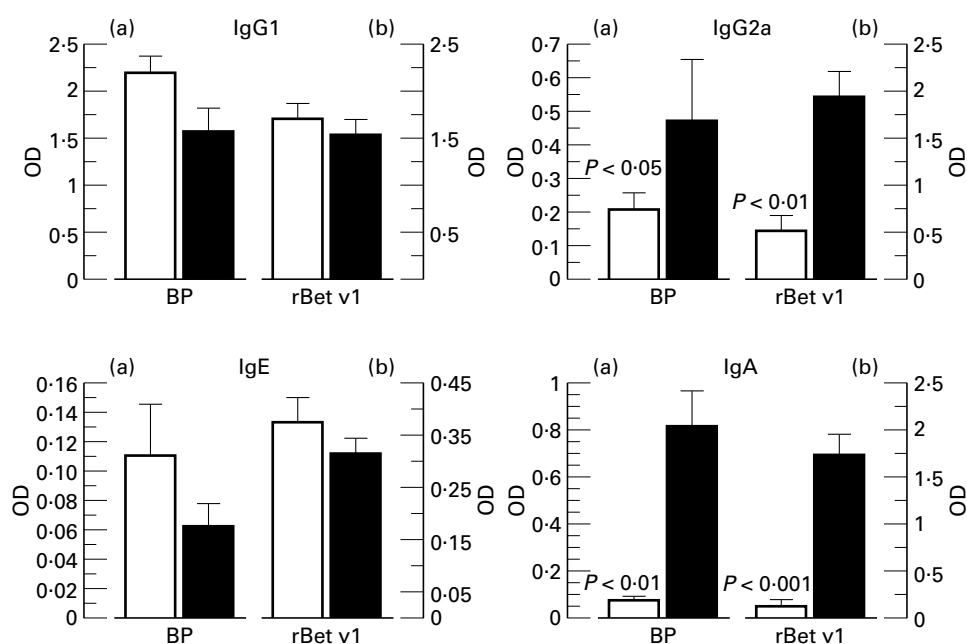


Fig. 2. Serum IgG1, IgG2a, IgE and IgA antibody responses in bronchial lavage against (a) birch pollen (BP) and (b) rBet v1. □, Mean optical density (OD) of six mice immunized intraperitoneally with rBet v1/Al(OH)₃ before inhalation with BP/cholera toxin (CT) (group 1); ■, mean OD of six mice aerosolized with BP/CT (group 2). Error bars show s.e.m., Mann–Whitney *U*-test.

consistently weaker or negative skin reactions to both BP and rBet v 1 allergen.

Neither in serum nor in BAL were antibodies to BP or rBet v 1 detected in mice which were either aerosolized with BP extract alone or with PBS. Accordingly, no positive skin reactions were elicited in these groups.

Representative skin sections from mice treated with Al(OH)₃, CT or without adjuvant are shown in Fig. 3.

Modulation of immune responses induced with Al(OH)₃ versus CT (group 3, group 4)

To investigate if the established Th2 and Th1 responses could be

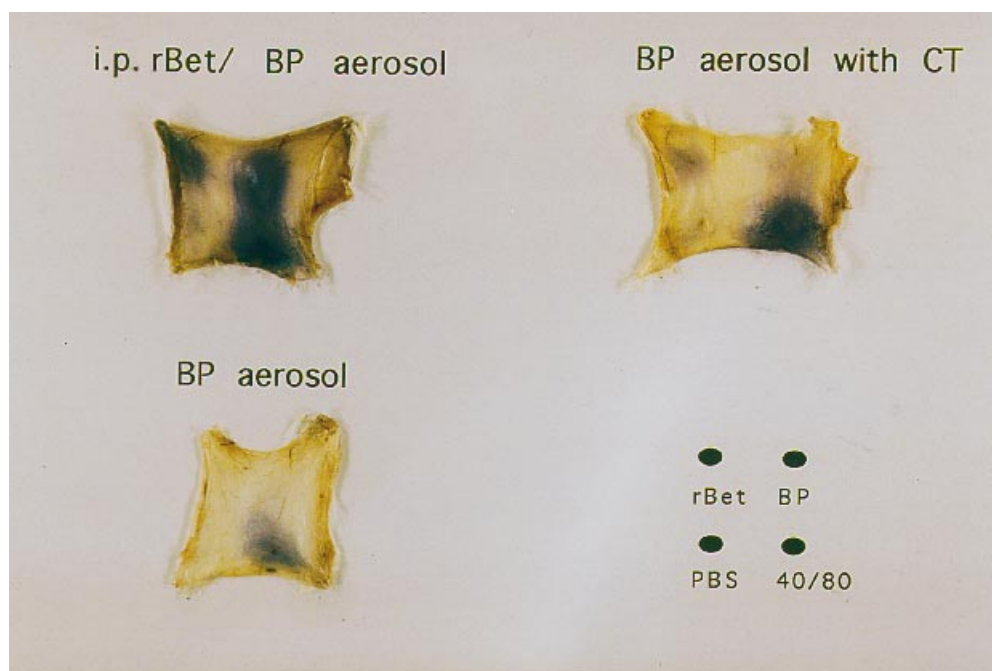


Fig. 3. Representative skin sections of type I skin reactions of mice treated with rBet v 1/Al(OH)₃ before birch pollen (BP) aerosol (group 1), BP/cholera toxin (CT) aerosol (group 2) or BP aerosol alone. The skin reactions to BP (2.5 µg/ml), rBet v 1 (2.5 µg/ml), histamine-releasing compound 48/80 (20 µg/ml) and PBS were measured at the inside of the abdominal skin 20 min after intradermal injection.

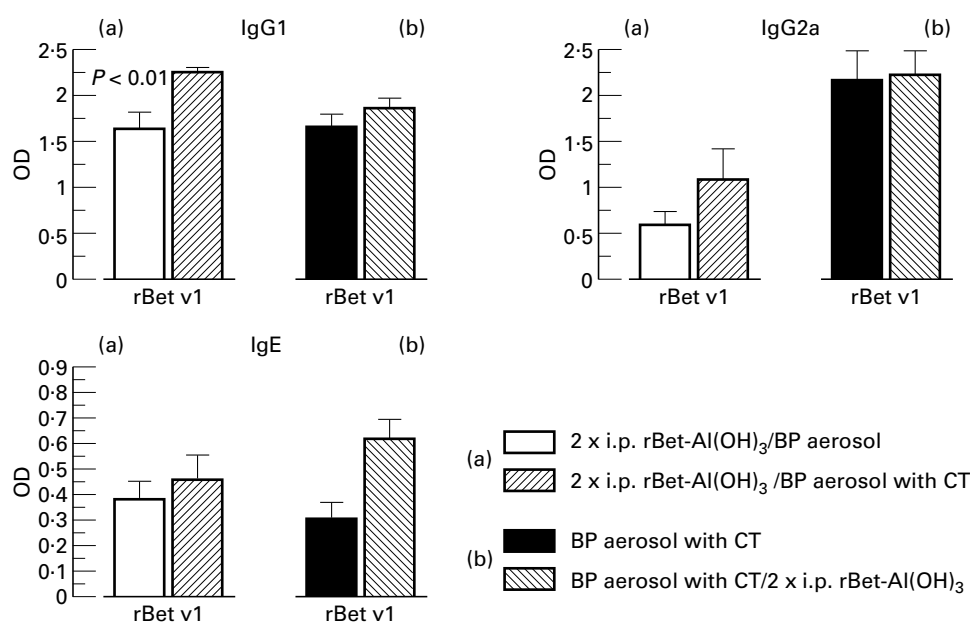


Fig. 4. Serum IgG1, IgG2a and IgE antibody responses to rBet v1. (a) Mean optical density (OD) of six mice treated with cholera toxin (CT)/birch pollen (BP) aerosol after rBet v1/Al(OH)₃ immunization (group 3) compared with group 1. (b) Mean OD of six mice immunized with rBet v1/Al(OH)₃ after CT/BP aerosol (group 4) compared with group 2. Error bars show s.e.m., Mann–Whitney *U*-test.

reversed, one group (group 3) was systemically immunized with rBet v 1/Al(OH)₃ and thereafter aerosolized with the CT/BP mixture, another (group 4) was systemically immunized with rBet v 1/Al(OH)₃ after aerosol inhalation of CT/BP.

The additional treatment with CT in the aerosol after systemic immunization (group 3) led to increased serum IgG1, IgG2a and IgE antibody levels to BP and rBet v 1 (serum IgG1 anti-rBet antibodies were significantly higher in group 3 (OD 2.3 (s.d. 0.12)) than in group 1 (OD 1.6 (s.d. 0.4)) $P = 0.005$) (Fig. 4a).

In group 4 the additional treatment led only to a slight increase in serum IgG2a, but a more pronounced increase (NS) in IgG1 and IgE antibody levels to BP and rBet v 1 compared with the CT group (group 2) (Fig. 4b).

Positive skin reactions could be observed in both group 3 and group 4.

Table 1. Proliferation and cytokine production in spleen cell cultures stimulated with birch pollen (BP)

Treatment	Stimulation index*	IFN- γ (pg/ml)	IL-4 (pg/ml)
Group 1	2.1 (0.2)	19 (45)	2.8 (0.24)
Group 2	4.2 (0.7)	3866 (2006)	3.9 (0.6)
Group 3	5.7 (2.1)	716 (731)	5.1 (1.1)
Group 4	19 (6.5)	3035 (239)	6.2 (1.6)

* Stimulation index, mean proliferation with antigen (ct/min)/medium control mean value (ct/min).

Group 1: immunized intraperitoneally with rBet/Al(OH)₃ before inhalation of BP extract; group 2: aerosolized with BP/cholera toxin (CT); group 3: injected intraperitoneally with rBet/Al(OH)₃ before BP/CT aerosol; group 4: BP/CT inhalation before rBet/Al(OH)₃ immunization.

Proliferative responses of spleen cells, IFN- γ and IL-4 production

The proliferative response of spleen cells after stimulation with BP was twice as high in the CT-treated group (group 2) than in the Al(OH)₃-preimmunized group (group 1, $P < 0.001$). Also, IFN- γ and IL-4 levels in the supernatants from BP-stimulated spleen cells were consistently higher in the CT-treated group than in the Al(OH)₃-preimmunized group ($P < 0.001$, Table 1). The ratio of IFN- γ /IL-4 was more than 140 times higher in the CT group than in the Al(OH)₃ group. This effect was due to enhanced IFN- γ rather than decreased IL-4 production (Table 1).

In group 3 the additional exposure to CT in the aerosol led to consistently higher allergen-specific proliferative responses in the spleen cell cultures ($P < 0.01$) than in the cultures of the Al(OH)₃-preimmunized group (group 1). Further, IFN- γ as well as IL-4 levels were significantly higher in the cultures of group 3 than in those of group 1 ($P < 0.05$), showing a shift towards a Th0 response. The IFN- γ /IL-4 ratio (20-fold increase) might, however, indicate a bias towards a Th1/Th0 response.

In group 4, systemic immunization with rBet v 1/Al(OH)₃ following the CT/BP inhalation (group 4) resulted in a four to five-fold increase in the proliferative response compared with group 2 ($P < 0.01$). There were significantly enhanced IL-4 levels in the spleen cell cultures of group 4 ($P < 0.01$), whereas IFN- γ levels did not markedly change. This led to a 50% reduction of the IFN- γ /IL-4 ratio compared with that after exclusive CT/BP inhalation (group 2).

DISCUSSION

Adjuvants are frequently used in conjunction with different vaccines and immunotherapeutic agents, such as in SIT of allergic diseases. Apart from the desired enhancing effects, it has been demonstrated that adjuvants also influence the quality of the immune response [33].

In the present study we compared the effects of $\text{Al}(\text{OH})_3$, an enhancer of IgE responses, and CT, a mucosal adjuvant, on the immune response to birch pollen and its major allergen Bet v 1. We demonstrated that systemic immunization of rBet v 1 in $\text{Al}(\text{OH})_3$ before aerosol inhalation of BP induced a Th2-like immune response, while inhalation of birch pollen mixed with CT induced a Th1-like response. In an attempt to reverse these ongoing immune responses we could achieve a shift towards a Th0-like pattern.

The use of adjuvants along with BP/rBet v 1 immunizations proved to be necessary, since aerosol inhalation of BP extract alone did not elicit an immune response. According to Holt *et al.*, inhalation of protein antigen induces a state of antigen-specific tolerance, preferentially directed against the IgE isotype [34,35]. In our model of BP inhalation the unresponsiveness to the allergen did not seem to be due to tolerogenic mechanisms, since subsequent systemic immunization with rBet v 1/ $\text{Al}(\text{OH})_3$ elicited IgG and IgE antibody responses (data not shown). Other antigens, such as ovalbumin (OA) or Der p (house dust mite allergen) might possess a stronger immunogenicity (or intrinsic activities), as these antigens have been successfully used for inhalative immunization without adjuvants [32,36].

It is known that parenteral administration of antigen with $\text{Al}(\text{OH})_3$ preferentially induces IgE synthesis in experimental animals [17,37]. Moreover, intraperitoneal administration of antigen has been shown to be an effective route for priming the respiratory mucosa [38,39]. In our model systemic (i.p.) immunization of rBet v 1 in $\text{Al}(\text{OH})_3$ and subsequent aerosol exposure to BP extract led to substantial IgG1/IgE antibody levels (Th2-type response, Fig. 2) and positive immediate-type skin reactions (Fig. 3), an immunological state comparable to that of human type I allergy.

CT is one of the most potent mucosal immunogens yet defined [20], enhancing the immune response to an antigen when simultaneously administered via the mucosal route [40]. In the present study CT (in combination with the allergen) was applied by aerosol inhalation. It has been suggested that CT enhances the immune response that is naturally induced by the antigen. Intranasal and/or oral administration of viral and bacterial antigens with CT preferentially induces Th1-like responses [29,41], whereas intranasal and/or oral administration of soluble protein antigens with CT promotes Th2-like immune responses [22,26]. Our results, however, are not consistent with this concept, since inhalation of BP with CT induced high levels of IgG2a antibodies, an isotype typical of a Th1 response (Fig. 2). The production of IgG2a antibodies is controlled by the Th1 cytokine, IFN- γ [7,42]. Indeed, spleen cell cultures of CT/BP-treated mice produced high levels of IFN- γ upon antigen-specific stimulation *in vitro* (Table 1). This shows that CT can also function as a potent stimulator of IFN- γ production for inhaled protein antigens. The levels of IgG1/IgE antibodies were decreased after CT treatment (though not statistically significant, Fig. 2). However, the type I skin reactions to BP and rBet v 1 were negative or very weak (Fig. 3). This might be explained by a direct effect of the high levels of IFN- γ on the mast cells, since this cytokine is known to inhibit differentiation and degranulation of mast cells in mice [43,44]. Taking these data together, we established a model of BP sensitization and demonstrated that the use of $\text{Al}(\text{OH})_3$ in combination with allergen inhalation led to immediate-type immune responses, comparable to type I allergy in humans. Bronchial administration of CT/allergen, on the other hand, systemically induced Th1-biased immune responses in this

model system. However, allergen-specific IgA antibodies in the respiratory mucosa, the actual site of allergen contact, could also be observed (Fig. 2). The induction of mucosal IgA antibodies in a Th1-dominated environment could be explained by the local occurrence of cytokines such as IL-10 or IL-6, described to provide signals for mucosal IgA regulation even in the absence of traditional Th2 cytokines [45].

SIT is the treatment of choice for human type I allergy. This treatment is performed by injecting subsequent doses of allergen and adjuvant, thereby modulating the allergen-specific T cell responses [46–48]. Based on our finding, we investigated whether CT/allergen inhalation could influence an ongoing allergic immune response. On the other hand, we tested if Th1 responses could be altered by immunization protocols leading to type I allergy in naive mice.

CT/BP aerosol subsequent to allergic sensitization (group 3) enhanced antibody production of all isotypes (Fig. 4a). *In vitro* production of both IL-4 and IFN- γ significantly increased, IFN- γ production, however, being more pronounced (20-fold increase in the IFN- γ /IL-4 ratio). The resulting Th response was similar to a Th1/Th0-like response. The fact that the skin reactions were not significantly reduced might be explained by the presence of already mast cell-bound IgE at the time of CT/BP administration.

Immunization with $\text{Al}(\text{OH})_3$ /allergen in Th1-biased animals (group 4) also altered the quality of the immune response. We observed an increase of all antibody isotypes (Fig. 4b) and enhanced IL-4 production (Table 1). The immune response could again be classified as Th0.

In the past practically all strategies to improve SIT were based on the specific interaction of the allergen with the immune system [15,16]. Therefore only few data exist promoting the use of selected adjuvants as concepts for the treatment of type I allergy [19,49,50]. In our opinion the use of $\text{Al}(\text{OH})_3$, which is conventionally applied during SIT, is even unsuited as adjuvant for injection immunotherapy in humans.

Our results suggest that the use of mucosal adjuvants might be of significance for immunotherapy of type I allergy. Further, we speculate that high levels of IgA antibodies at the site of allergen contact (respiratory mucosa) might have protective capacity. In fact, therapeutic strategies for type I allergy targeting the mucosa-associated lymphoid tissues (MALT) have been shown to be effective [51,52]. It remains to be investigated if modified [53] or other forms of mucosal adjuvants [54,55] could be promising candidates for the prophylaxis as well as modulation of allergic immune responses to inhaled allergens.

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