

# Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy

Ursula Wiedermann<sup>1</sup>, Beatrice Jahn-Schmid<sup>1,2</sup>, Marianne Lindblad<sup>3</sup>, Carola Rask<sup>3</sup>, Jan Holmgren<sup>3</sup>, Dietrich Kraft<sup>1</sup> and Christof Ebner<sup>1</sup>

<sup>1</sup>Division of Immunopathology, Institute of General and Experimental Pathology, University of Vienna, Waehringuer Guertel 18–20, 1090 Vienna, Austria

<sup>2</sup>Centre of Ultrastructural Research, University of Agriculture, Vienna, Austria,

<sup>3</sup>Department of Microbiology and Immunology, University of Göteborg, Göteborg, Sweden

**Keywords:** aerosol, BALB/c, cholera B subunit, intranasal, immunomodulation, T<sub>H</sub>2 response

## Abstract

Recent reports have demonstrated that feeding small amounts of antigen conjugated to the B subunit of cholera toxin (CTB) suppress immune responses in experimental models of certain T<sub>H</sub>1-based autoimmune diseases. We have established a model of aerosol sensitization leading to T<sub>H</sub>2-mediated allergic immune responses in BALB/c mice. In the present study two different antigens, the dietary antigen ovalbumin (OVA) and the inhalant allergen Bet v 1 (the major birch pollen allergen), chemically coupled to recombinant CTB were tested for their potential to influence T<sub>H</sub>2-like immune responses. Intranasal administration of OVA–CTB prior to sensitization with OVA led to a significant decrease of antigen-specific IgE antibody levels, but a marked increase of OVA-specific IgG2a antibodies as compared to non-pretreated, sensitized animals. Antigen-specific lympho-proliferative responses *in vitro* were reduced by 65% in the pretreated group; IL-5 and IL-4 production were decreased in responder cells of lungs and spleens of nasally pretreated mice. In contrast, mucosal administration of rBet v 1–CTB conjugates prior to sensitization led to an up-regulation of allergen-specific IgE, IgG1 and IgG2a, increased *in vitro* lympho-proliferative responses as well as augmented production of IL-5, IL-4, IL-10 and IFN- $\gamma$ . Intranasal administration prior to sensitization of unconjugated allergens showed also contrasting effects: OVA could not significantly influence antigen-specific antibody or cytokine production, whereas intranasal pretreatment with unconjugated Bet v 1 suppressed allergen-specific immune responses *in vivo* and *in vitro*. These results demonstrated that the two antigens—in conjugated as in unconjugated form—had different effects on the T<sub>H</sub>2 immune responses. We therefore conclude that the tolerogenic or immunogenic properties of CTB—and probably also other antigen-delivery systems—strongly depend on the nature of the coupled antigen–allergen.

## Introduction

It is well established that mucosal administration of soluble antigens induces systemic immunological unresponsiveness, a phenomenon known as mucosal tolerance (1,2). However, it has been recognized that the effectiveness of mucosal tolerance often requires repeated and large amounts of antigen, and sometimes the suppressed immune responses are of short duration. On the other hand, molecules with

known mucosa-binding properties are known to induce local and systemic immune responses when administered by the mucosal routes (3). Among these, cholera toxin (CT) is one of the most potent mucosal immunogens, which, when administered simultaneously with an antigen by the mucosal route, enhances immune responses to the co-administered antigen (4). In contrast to cholera holotoxin, mucosal adminis-

tration of only the non-toxic, mucosa-binding B subunit of CT (CTB), physically coupled to an antigen, has been recently recognized to enhance peripheral tolerance induction (5). Sun *et al.* demonstrated that a single feeding of antigen conjugated to CTB led to suppression of T cell responses *in vivo* and *in vitro* at doses 15- to 500-fold lower than at those of corresponding regimes using unconjugated antigens (5). In line with this finding further studies have demonstrated the effectiveness of such antigen-CTB formulations in preventing or treating certain  $T_H1$ -based diseases, such as experimental autoimmune encephalomyelitis (6) or spontaneous autoimmune diabetes (7). Recently, a similar transmucosal carrier-delivery system, the enterotoxin B of *Escherichia coli* conjugated to antigen, has been successfully used for suppression not only of antigen-specific T cell responses but also of antigen-specific IgE production (8). So far, this is the only study providing evidence that such a mucosal antigen-delivery system might be a promising strategy to suppress immune responses, based on excessive  $T_H2$  cell activity.

Type I allergy is very common in areas of the temperate climate zone and the prevalence of the disease has constantly increased within the last years (9). This genetically determined immunodisorder, manifested by symptoms like allergic rhinitis, conjunctivitis or allergic asthma, is based on the excessive production of IgE antibodies against allergenic molecules (10). The pathway of IgE regulation is well described for mice and humans, and essentially explained by the reciprocal activity of IL-4 and IL-5, mediating  $T_H2$  responses, and IFN- $\gamma$ , which antagonizes these effects (11–13).

We have established a murine model of aerosol sensitization leading to allergen-specific  $T_H2$ -mediated immune responses i.e. high IgE/IgG1 versus low IgG2a antibody levels and positive type I skin tests *in vivo*, an immunological state comparable to that of human type I allergy. Using this model system we have recently reported that simultaneous inhalation of allergen and cholera holotoxin can modulate the allergic immune response in naive as well as in sensitized animals (14).

In the present study we investigated, whether mucosal delivery of antigen-CTB conjugates could also be successfully used to suppress type I allergic immune responses. Two different antigens were used for this purpose: ovalbumin (OVA), a frequently experimentally used dietary antigen, and recombinant Bet v 1, the major allergen of birch pollen (BP) and a clinically important inhalant allergen (15,16).

## Methods

### Animals

Female, 7-week-old, BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Science and Research.

### Antigens

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

BP was obtained from Allergon (Engelholm, Sweden) and

used for the preparation of a BP extract as previously described (14).

Recombinant CTB was produced in a mutant strain of *Vibrio cholerae* deleted from the CTA subunit gene and transfected with a plasmid encoding CTB. CTB was purified by sequential precipitation and gel filtration chromatography (17,18). OVA (Sigma, St Louis, MO) and rBet v 1 were covalently bound to CTB at a molar ratio of 1:1 using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as bifunctional coupling reagent, according to the protocol described by Sun *et al.* (5). The resulting OVA-CTB and rBet v 1-CTB conjugates were purified by gel filtration through a column of Sephacryl S-300. The conjugates were shown to retain GM1 binding capacity and serological activity of both OVA and rBet v 1 by means of solid-phase ELISA (19).

### Nasal administration of CTB-coupled proteins

Mice ( $n = 6$ /group) were anesthetized and 40  $\mu$ g/30  $\mu$ l NaCl of the OVA-CTB (20  $\mu$ g OVA conjugated to 20  $\mu$ g CTB) or rBet v 1-CTB (20  $\mu$ g rBet v 1 coupled to 20  $\mu$ g CTB) were intranasally applied. For control purposes corresponding amounts of unconjugated OVA or rBet v 1 or NaCl were intranasally applied to mice ( $n = 6$ /group). This procedure was performed at three occasions at 7 day intervals prior to sensitization (days -21, -14 and -7; Fig. 1 experimental design).

### Sensitization

Pretreated and sham-pretreated mice were once injected i.p. with 50  $\mu$ g OVA or 1  $\mu$ g rBet v 1 adsorbed to  $Al(OH)_3$  (day 0). Fourteen days later aerosol exposure to a 0.1% OVA or BP solution was daily performed during a period of 2 times over 5 days (days 14–18 and 21–25) as previously described (14). The OVA and the BP solution were aerosolized using a nebulizer (nebulizer 646; Devilbiss, Somerset, PA). The mice were exposed to 4 ml solution for 20 min/day (Fig. 1, experimental design).

### Sampling

Blood samples were taken before sensitization and 6 days after the last aerosol exposure. Serum was prepared and stored at -20° C until analyzed.

Bronchoalveolar lavage (BAL) was collected by lavaging the lungs with 1 ml PBS inserted through a small incision in the trachea of the sacrificed mice (14).

### Immunoblots

Proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting according to standard protocols (20,21). Sera from OVA and Bet v 1/BP immunized mice were incubated in 1/5 (IgE) and 1/100 (IgG1 and IgG2a) dilutions with nitrocellulose stripes coated with OVA or BP extract. Immunoblots were performed with rat anti-mouse IgE (1/250), IgG1 (1/500) or IgG2a (1/250; PharMingen, San Diego, CA) followed by radiolabeled sheep anti-rat Ig (1/1000; Amersham, Little Chalfont, UK).

### Determination of antigen-specific antibody levels in serum and bronchial lavage

Microtiter plates (Nunc, Roskilde, Denmark) were coated with OVA (10  $\mu$ g/ml carbonate buffer) and BP extract (50  $\mu$ g/ml).

## Experimental design

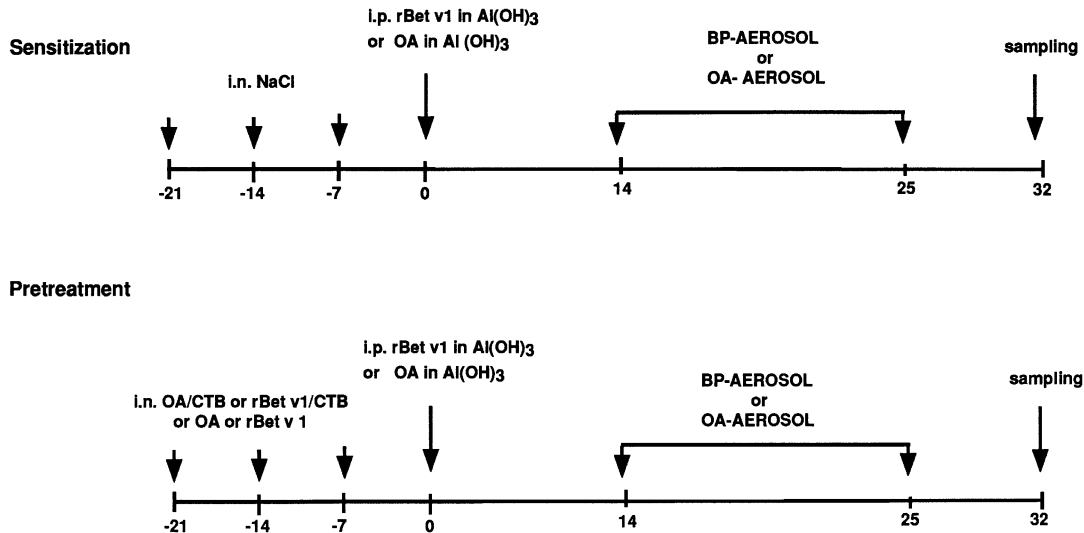


**Fig. 1.** Experimental design. Two groups of control mice ( $n = 6/\text{group}$ ) were either i.p. immunized with OVA/Al(OH)<sub>3</sub> or with rBet v 1/Al(OH)<sub>3</sub> and subsequently aerosolized with an 0.1% OVA or BP solution. Before sensitization 0.9% NaCl (sham-pretreatment) was intranasally applied. Four groups of mice ( $n = 6/\text{group}$ ) were 3×intranasally treated with OVA-CTB, rBet v 1-CTB, unconjugated OVA or unconjugated rBet v 1. Thereafter the mice were immunized with OVA or rBet v 1/BP as described for the control mice.

Serum samples were diluted 1/1000 for IgG1, 1/500 for IgG2a and 1/10 for IgE; BAL was used undiluted. Rat anti-mouse IgG1, IgG2a, IgE and IgA antibodies (1 µg/ml; PharMingen) and thereafter peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson ImmunoResearch, Westgrove, PA) were used. Color development was performed as previously described (14).

### Lymphocyte proliferation assay

At sacrifice, 7 days after the last aerosol exposure (day 32), spleens and lungs with associated lymph nodes were removed under sterile conditions. The organs were homogenized, erythrocytes were lysed, and the cells washed and resuspended in complete medium (RPMI, 10% FCS, 0.1 mg/ml gentamycin, 2 mM glutamine and 50 µM 2-mercaptoethanol). Suspensions of spleen cells as well as lung cells with associated lymph nodes were plated into 96-well round-bottom plates at a concentration of  $2 \times 10^5/200 \mu\text{l}/\text{well}$  and stimulated with and without concanavalin A (Con A; 0.5 µg/well), OVA (40 µg/well) or BP (5 µg/well) for 4 days. Thereafter the cultures were pulsed with 0.5 µCi/well [<sup>3</sup>H]thymidine (Amersham) for 16 h and the proliferative responses measured by scintillation counting.

### Measurement of cytokine production in spleen and lung cell cultures

For determination of IL-5, IL-4, IL-10 and IFN-γ production spleen and lung cells were cultured with and without Con A (2.5 µg/well), OVA (100 µg/well) and BP (25 µg/well) in

48-well plates (Costar, Cambridge, MA) at a concentration of  $5 \times 10^6/0.5 \text{ ml}/\text{well}$ . After 24 and 48 h supernatants were taken and stored at -20° C until analyzed.

The levels of IL-5, IL-4, IL-10 and IFN-γ were measured with mouse ELISA kits (Endogene, Cambridge, MA). The sensitivity of the IL-5 and IL-4 assay was <5 pg/ml and of the IL-10 and IFN-γ ELISA <15 pg/ml.

### Statistics

For statistical analysis the Mann-Whitney *U*-test was used.

## Results

### Sensitization

Mice, immunized i.p. with OVA or Bet v 1 adsorbed to Al(OH)<sub>3</sub> and subsequently exposed to an OVA or BP solution via aerosol, displayed high allergen-specific IgG1/IgE versus low IgG2a antibody levels. Figure 2(A) demonstrates the binding of serum IgE, IgG1 and IgG2a antibodies from OVA-sensitized mice to the 40 kDa molecule. Mouse sera from Bet v 1/BP immunized mice only bound to the 17 kDa Bet v 1 molecule (Fig. 2B). Therefore, all tests performed with BP extract reflect the immune reactivity to Bet v 1 exclusively.

*Mucosal administration of OVA-CTB conjugate leads to suppression of IgE antibody levels whereas application of rBet v 1-CTB conjugates enhances antibody responses of all isotypes*

Antigen-specific antibody levels in serum and BAL were measured in mice treated 3 times intranasally with OVA-CTB

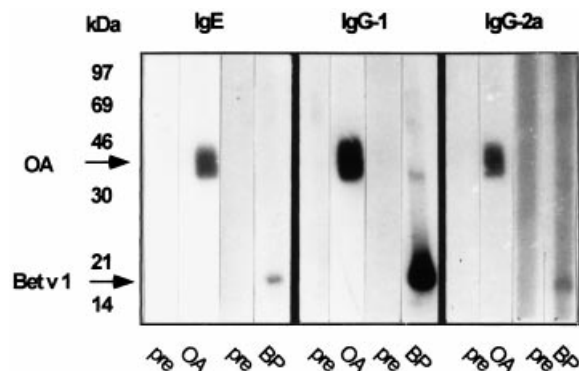


**Fig. 2.** Immunoblot, performed with pooled sera of mice immunized according to the standard immunization protocol, i.e.  $1 \times i.p.$  injection of  $Al(OH)_3$  adsorbed OVA or Bet v 1, followed by aerosol exposure to 0.1% OVA or BP solution. (pre) Nitrocellulose stripes incubated with pooled preimmune sera. (OVA) Binding of serum IgE, IgG1 and IgG2a from OVA sensitized mice to the 40 kDa OVA molecule. (BP) Binding of serum IgE, IgG1 and IgG2a of Bet v 1/BP immunized mice to Bet v 1, the 17 kDa major BP allergen. Additional IgG1 binding to the 34 kDa Bet v 1 dimer.

or unconjugated OVA or 3 times intranasally pretreated with rBet v 1-CTB conjugates or unconjugated rBet v 1 and compared with those of OVA- or Bet v 1-sensitized control mice.

As shown in Fig. 3(A) mucosal pretreatment with OVA-CTB conjugates led to a significant reduction of OVA-specific IgE antibody levels ( $P < 0.05$ ) but simultaneously to a drastic increase of anti-OVA IgG2a antibody levels ( $P < 0.01$ ). IgG1 anti-OVA antibody levels (Fig. 3 A) as well as IgA anti-OVA levels in BAL did not differ between the sensitized (IgA-OD:  $0.279 \pm 0.23$ ) and the nasally pretreated mice (IgA-OD:  $0.219 \pm 0.219$ ). Intranasal administration of equivalent doses of unconjugated OVA could not markedly decrease antigen-specific IgE nor significantly increase IgG2a antibody levels (Fig. 3B).

In total contrast to the pretreatment with OVA-CTB conjugates, intranasal administration of rBet v 1-CTB conjugate prior to sensitization led to enhanced antibody responses of all isotypes examined. In particular, IgE, IgG1 and IgG2a antibodies against BP/Bet v 1 were significantly higher in the conjugate pretreated group as compared to the sensitized animals ( $P < 0.01$ , Fig. 3A). A similar, although not significant increase in allergen-specific IgA antibody levels in BAL was noted in the conjugate pretreated animals (OD:  $1.0 \pm 0.7$ ) compared to the controls (OD:  $0.087 \pm 0.05$ ). To investigate, if the enhanced immune responses were due to CTB, unconjugated rBet v 1 was intranasally applied before sensitization. This pretreatment led to a strong reduction of the allergen-specific antibody responses of all isotypes as shown in Fig. 3(B).

*Pretreatment with OVA-CTB conjugates leads to suppression, whereas rBet v 1-CTB conjugates cause enhancement of antigen-specific lympho-proliferative responses in vitro*

Antigen-specific lympho-proliferative responses were measured in lung and spleen cell cultures of mice pretreated with antigen-CTB conjugate or unconjugated antigen and

compared with cultures derived from sensitized animals. Lympho-proliferative responses in lung cells of OVA-CTB pretreated mice were reduced by 65% [stimulation index (SI):  $2 \pm 1.0$ ] compared to the sensitized mice (SI:  $5.7 \pm 4.7$ ,  $P < 0.05$ ). An even stronger reduction (81%) of the lympho-proliferative response was noted in the group of mice pretreated with unconjugated OVA (SI:  $2.1 \pm 2.3$ ) compared to the sensitized mice (SI:  $11.3 \pm 4.4$ ,  $P < 0.02$ ). However, in spleen cell cultures no differences in the antigen-specific proliferative responses were observed between the groups (Fig. 4).

In contrast, intranasal administration of rBet v 1-CTB prior to sensitization with BP led to a 273% increase of lympho-proliferative responses of lung cell cultures (SI:  $5.6 \pm 5.2$ ) and a 163% enhanced response of spleen cells (SI:  $5.1 \pm 3.5$ ) as compared to the sensitized animals (SI:  $1.5 \pm 0.7$  respectively SI:  $1.9 \pm 0.3$ ) (Fig. 4).

However, intranasal administration of unconjugated Bet v 1 resulted in a 37–44% reduction of allergen-specific lympho-proliferative responses in lung (SI:  $1.5 \pm 0.2$ ) and spleen cells (SI:  $2.0 \pm 0.3$ ) as compared to the sensitized animals (SI:  $2.4 \pm 0.9$  respectively SI:  $3.6 \pm 1.7$ ) (Fig. 4).

*Pretreatment with OVA-CTB decreases production of IL-5 and IL-4, whereas rBet v 1-CTB leads to increased cytokine production in vitro*

*In vitro* production of IL-5, IL-4, IL-10 and IFN- $\gamma$  was measured in spleen and lung cell cultures of sensitized and pretreated animals. Intranasal administration of OVA-CTB conjugates prior to sensitization led to a 2.2- to 7.6-fold reduction of IL-5 and a 1.4-fold reduction of IL-4 levels in cultures of pooled spleen and lung cells compared to the sensitized controls (Table 1). IFN- $\gamma$  and IL-10 levels did not markedly differ between the groups. No significant differences in IL-5 and IFN- $\gamma$  production were observed in cell cultures of mice pretreated with unconjugated OVA compared to OVA sensitized mice (Table 1).

In contrast, pretreatment with rBet v 1-CTB led to a drastic increase of IL-5, IL-4, IFN- $\gamma$  and IL-10 production in spleen and lung cell cultures compared to the cytokine levels measured in cultures of the sensitized control animals (Table 1). This augmented immune response *in vitro* was consistent with the enhanced allergen-specific antibody responses *in vivo* (Fig. 3).

The opposite effects were obtained from mice intranasally treated with unconjugated Bet v 1 prior to sensitization. In line with the suppressed antibody production (Fig. 4) also the *in vitro* production of IL-5, IL-4, IL-10 and IFN- $\gamma$  was markedly reduced compared to that of the sensitized animals (Table 1).

## Discussion

In the present study we demonstrated that the use of antigen-CTB conjugates had opposite effects on  $T_H2$ -like immune responses depending on the antigen coupled to CTB. Intranasal administration of OVA-CTB conjugates prior to sensitization suppressed, whereas administration of rBet v 1-CTB conjugates stimulated immune responses *in vivo* and *in vitro*.

Previous reports have demonstrated that mucosal administration of soluble antigens conjugated to CTB is more effective than that of unconjugated antigens to induce peripheral

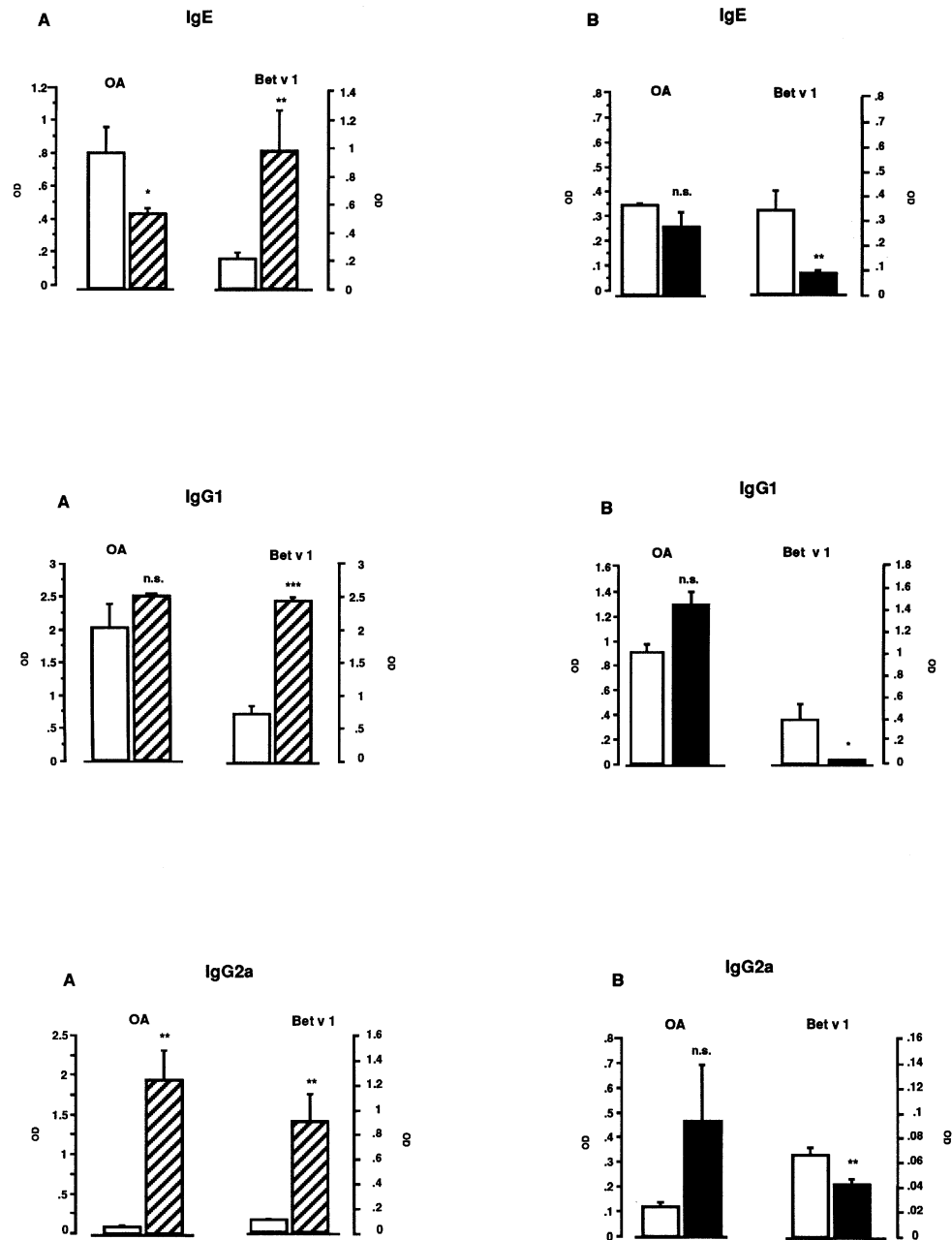


**Fig. 3.** Serum IgE, IgG1 and IgG2a antibody responses against OVA or Bet v 1 measured by ELISA. (A) White bars represent the mean OD of six control mice immunized with OVA or Bet v 1/BP according to the standard protocol. Hatched bars represent the mean OD of six mice, intranasally treated with OVA-CTB or rBet v 1-CTB prior to sensitization. (B) White bars represent the mean OD of six control mice immunized with OVA or Bet v 1/BP. Black bars represent the mean OD of six mice, intranasally treated with unconjugated OVA or unconjugated rBet v 1 prior to sensitization. Error bars show SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , Mann-Whitney  $U$ -test.

tolerance in naive as well as sensitized animals. These studies, mainly performed in models of  $T_H1$ -mediated diseases, consistently reported about the ability of antigen-CTB conjugates to reduce delayed-type hypersensitivity reactions or  $T$  cell responses *in vitro* (5,6,7,22).

Less data exist about the suppressive effects on  $T_H2$ -like immune responses. A recent study showed that CTB conjugated to the *Leishmania* antigen LACK or OVA selectively reduced  $T_H1$  responses (IFN- $\gamma$  production), whereas  $T_H2$

responses (IL-4/IL-5 production) remained unaffected by this treatment. It was therefore suggested that the selective tolerance of  $T_H1$  cells was due to a common effect of CTB rather than an antigen-specific effect of the proteins conjugated to CTB. The authors concluded that such therapeutic strategies might therefore preferentially be used for treatment of  $T_H1$ -based immunopathological situations (22). However, another study recently reported that a similar antigen delivery system—the bacterial product LTB coupled to OVA—could

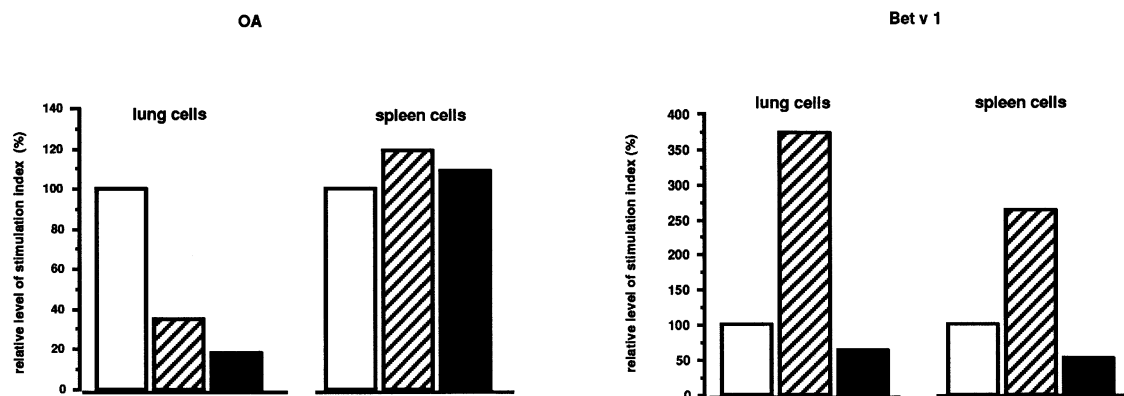


**Fig. 4.** Relative levels of stimulation indices of responder cells from lung and spleen cell cultures, stimulated with OVA or BP antigen. The white bars represent the proliferative response of sensitized mice, hatched bars of antigen-CTB pretreated mice and black bars of mice pretreated with unconjugated antigen prior to sensitization. The stimulation index of the sensitized mice was set to 100% and the stimulation indices of the pretreated groups were expressed as relative values.

**Table 1.** *In vitro* cytokine production in spleen and lung cell cultures

| Spleen cells |               |                      |                      |                      |                        |
|--------------|---------------|----------------------|----------------------|----------------------|------------------------|
| Pretreatment | Sensitization | IL-5                 | IL-4                 | IL10                 | IFN- $\gamma$          |
| NaCl         | OVA           | 39.6                 | 4.8                  | 8.9                  | n.d.                   |
| OVA/CTB      | OVA           | 18                   | 5                    | 19.7                 | n.d.                   |
| NaCl         | Bet v 1/BP    | 2.3 $\pm$ 0.9        | 10.3 $\pm$ 7.2       | 72.3 $\pm$ 25.2      | 75.9 $\pm$ 48.0        |
| rBet v 1/CTB | Bet v 1/BP    | 141.2 $\pm$ 115.5**  | 28.2 $\pm$ 22.1 n.s. | 319.8 $\pm$ 193**    | 1517.9 $\pm$ 10633**   |
| NaCl         | OVA           | 68.8 $\pm$ 10.7      | n.d.                 | n.d.                 | 309.0 $\pm$ 370.2      |
| OVA          | OVA           | 56.9 $\pm$ 67.8 n.s. | n.d.                 | n.d.                 | 234.8 $\pm$ 414.3 n.s. |
| NaCl         | Bet v 1/BP    | 81.7 $\pm$ 170       | 44 $\pm$ 97.2        | 108 $\pm$ 65.16      | 1041.6 $\pm$ 931.3     |
| rBet v 1     | Bet v 1/BP    | 2.4 $\pm$ 4.1*       | 1.7 $\pm$ 2.4 n.s.   | 68.5 $\pm$ 49.5 n.s. | 17.1 $\pm$ 14.5**      |
| Lung cells   |               |                      |                      |                      |                        |
| Pretreatment | Sensitization | IL-5                 | IL-4                 | IL10                 | IFN- $\gamma$          |
| NaCl         | OVA           | 86.5                 | 8.1                  | 18.9                 | 90                     |
| OVA/CTB      | OVA           | 11.4                 | 4.7                  | 20                   | 120                    |
| NaCl         | Bet v 1/BP    | 22.2 $\pm$ 28        | 5.7 $\pm$ 13         | 5.3 $\pm$ 9          | 7.7 $\pm$ 11.8         |
| rBet v 1/CTB | Bet v 1/BP    | 135 $\pm$ 197*       | 3.3 $\pm$ 3.9        | 56.8 $\pm$ 39**      | 103.5 $\pm$ 110        |
| NaCl         | OVA           | 272 $\pm$ 53.3       | n.d.                 | n.d.                 | 7.7 $\pm$ 11.8         |
| OVA          | OVA           | 163 $\pm$ 197 n.s.   | n.d.                 | n.d.                 | 62.5 $\pm$ 114.5 n.s.  |

Concentrations of cytokines in supernatants of spleen- and lung cell cultures ( $n = 6/\text{group}$ ) of mice intranasally pretreated or sham- (NaCl) pretreated before sensitization. IL-5 and IL-4 production (pg/ml) was measured after 24 hours, IL-10 and IFN- $\gamma$  (pg/ml) after 48 hours stimulation with antigen. Values represent the mean of 6 individual cell cultures of each group ( $\pm$  standard deviation). In the experiment of OVA/CTB pretreated and OVA sensitized mice spleen and lung cells were pooled within each group. n.d. = not done, n.s. = not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

suppress both  $T_H1$  and  $T_H2$  responses, as demonstrated in a decreased delayed-type hypersensitivity reaction as well as suppressed OVA-specific IgE antibody responses (8).

In the present study we used our murine model of aerosol sensitization to investigate the potential of two different antigens, the dietary antigen OVA and the inhalant allergen Bet v 1, coupled to CTB for their potential to affect  $T_H2$ -like immune responses. Our present data demonstrated that intranasal pretreatment with OVA-CTB conjugates led to a significant

reduction of OVA-specific IgE antibody levels. We also noted a significant increase in antigen-specific IgG2a antibody production, indicating the capacity to drive the immune response towards a  $T_H1$ -like response (Fig. 3A). The reciprocal activities of IL-5/IL-4—crucial for induction of  $T_H2$  responses—and IFN- $\gamma$  known to control the production of IgG2a in the murine system—is well documented (12,23). In accordance with the antibody responses the *in vitro* synthesis of IL-5/IL-4, but not of IFN- $\gamma$ , was decreased in lung and spleen

cell cultures of OVA-CTB pretreated mice (Table 1). This immunomodulating effect was due to the CTB component of the conjugate, since intranasal application of comparable amounts of unconjugated OVA could not significantly reduce IgE or enhance IgG2a antibody levels (Fig. 3B), nor significantly modulate the cytokine production profile (Table 1). Our data indicates that  $T_H2$  responses can be influenced by intranasal administration of OVA-CTB. This is in contrast to the study of McSorely *et al.*, reporting that pretreatment with OVA-CTB conjugates via the same route failed to affect  $T_H2$  immune responses (22).

Notably, using rBet v 1-CTB conjugates under identical experimental conditions resulted in a general stimulation of allergen-specific antibody production of all isotypes in serum (Fig. 3A) and a concomitant increase of IgA in BAL. T cell proliferative responses and the cytokine production *in vitro* (IL-5, IL-4, IFN- $\gamma$  and IL-10) were also significantly enhanced in cell cultures (Fig. 4 and Table 1). In contrast to Bet v 1 coupled to CTB, intranasal application of unconjugated Bet v 1 led to a significant reduction of antibody responses of all isotypes (Fig. 3B), T cell proliferation (Fig. 3B) as well as cytokine production *in vitro* (Table 1). These observations demonstrated that, in the case of Bet v 1, CTB acted as an efficient adjuvant.

It has been demonstrated that the method of conjugation can influence the effect of such a transmucosal delivery system in terms of suppression or stimulation of the immune responses. These different effects have been explained by possible alterations of the antigen structure due to different methods of conjugation (8,24). Since we used the same method of conjugation for both antigens, our study indicates that the nature of the antigen *per se* influenced the quality of the immune response. It has been suggested that certain allergens have intrinsic abilities—in addition to acting as an immunogen—to intervene with regulatory processes involved in the development of allergy. Such intrinsic activities can be due to enzymatic properties (25–27) and/or certain molecular/structural features of the allergen. In the latter respect, Bet v 1 shows conformational epitopes, which can be destroyed by producing two parts of the molecule (28), whereas OVA is known to possess only continuous epitopes (29). These structural differences of the two molecules might have been responsible for the different outcome of the treatment with conjugated as well as unconjugated Bet v 1 compared to OVA.

In conclusion, considering CTB as a potential antigen-delivery system in  $T_H2$ -based immunopathological conditions—such as type I allergy—the above described formulation might only be successfully applicable with certain allergens. Consequently, the effects of novel adjuvants/antigen-delivery systems cannot be generalized based on experiments performed with model antigens, but need to be individually evaluated.

## Acknowledgements

The excellent technical assistance of Ms Renate Steiner-Göttl and Karin Baier is very much appreciated. The study was supported by grants from the Austrian Science Foundation (P 12889, S 7206-MOB), ALK, Copenhagen, Denmark and the Swedish Medical Research Council.

## Abbreviations

|         |                             |
|---------|-----------------------------|
| BAL     | bronchoalveolar lavage      |
| BP      | birch pollen                |
| Bet v 1 | major birch pollen allergen |
| Con A   | concanavalin A              |
| CT      | cholera toxin               |
| CTA     | cholera toxin A subunit     |
| CTB     | cholera toxin B subunit     |
| OVA     | ovalbumin                   |
| r       | recombinant                 |
| SI      | stimulation index           |

## References

- Mowat, A. M. 1994. Oral tolerance and regulation of immunity to dietary antigens. In Ogra, P., Mestecky, J., Lamm, M., Strober, W., McGhee, J. and Bienenstock, J., eds, *Handbook of Mucosal Immunology*, p. 185. Academic Press, San Diego, CA.
- Weiner, H. L. 1997. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today* 18:335.
- De Aizpurua, H. J. and Russel-Jones, G. J. 1988. Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding. *J. Exp. Med.* 167:440.
- Elson, C. O. and Eadling, W. 1984. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* 132:2736.
- Sun, J. B., Holmgren, J. and Czerkinsky, C. 1994. Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. *Proc. Natl Acad. Sci. USA* 91:10795.
- Sun, J. B., Rask, C., Olsson, T., Holmgren, J. and Czerkinsky, C. 1996. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc. Natl Acad. Sci. USA* 93:7196.
- Bergerot, I., Ploix, C., Petersen, J., Moulin, V., Rask, C., Fabien, N., Lindblad, M., Mayer, A., Czerkinsky, C., Holmgren, J. and Thivolet, C. 1997. A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. *Proc. Natl Acad. Sci. USA* 94:4610.
- Tamura, S., Hatori, E., Tsuruhara, T., Aizawa, C. and Kurata, T. 1997. Suppression of delayed -type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin. *Vaccine* 15:225.
- Wuethrich, B., Schindler, C., Leuenberger, P. and Ackermann-Liebrich, U. 1995. Prevalence of atopy and pollinosis in the adult population of Switzerland (Sapaldia study). *Int. Arch. Allergy. Immunol.* 106:149.
- Casolaro, V., Georas, N., Song, Z. and Ono, S. J. 1996. Biology and genetics of atopic disease. *Curr. Opin. Immunol.* 8:796.
- Fiorentino, D. F., Bond, M. W. and Mosmann, T. R. 1989. Two types of mouse helper T cells. IV.  $T_H2$  clones secrete a factor that inhibits cytokine production by  $T_H1$  cells. *J. Exp. Med.* 170:2081.
- Mosmann, T. R. and Coffman, R. L. 1989.  $TH1$  and  $TH2$  cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145.
- Romagnani, S. 1994. Regulation of the development of type 2 T-helper cells in allergy. *Curr. Opin. Immunol.* 6:838.
- Wiedermann, U., Jahn-Schmid, B., Fritsch, R., Bauer, L., Renz, H., Kraft, D. and Ebner, C. 1998. Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a  $T_H1$ -like immune response to Bet v 1, the major birch pollen allergen. *Clin. Exp. Immunol.* 111:144.
- Ipsen, H. and Loewenstein, H. 1983. Isolation and characterization of the major allergen of birch pollen (*Betula verrucosa*). *J. Allergy Clin. Immunol.* 72:150.
- Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Scheiner, O. and Kraft, D. 1989. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 44:385.

- 17 Sanchez, J. and Holmgren, J. 1989. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA* 86:481.
- 18 Lebens, M., Johansson, S., Osek, J., Lindblad, M. and Holmgren, J. 1993. Large-scale production of *Vibrio cholerae* toxin B subunit for use in oral vaccines. *Biotechnology* 11:1574.
- 19 Svennerholm, A.M. and Holmgren, J. 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. *Curr. Microbiol.* 1:19.
- 20 Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350.
- 21 Jarolim, E., Tejkl, M., Rohac, M., Schlerka, G., Scheiner, O., Kraft, D., Breitenbach, M. and Rumpold, H. 1989. Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. *Int. Arch. Allergy Immunol.* 90:54.
- 22 McSorley, S. J., Rask, C., Pichot, R., Julia, V., Czerkinsky, C. and Glaichenhaus, N. 1998. Selective tolerization of T<sub>H</sub>1-like cells after nasal administration of a cholera toxoid-LACK conjugate. *Eur. J. Immunol.* 28:424.
- 23 Snapper, C. M. and Paul, W. E. 1987. Interferon gamma and B cell stimulatory factor I reciprocally regulate Ig isotype production. *Science* 236:944.
- 24 Stok, W., van der Heijden, P. J. and Bianchi, A. T. J. 1994. Conversion of orally induced suppression of the mucosal immune response to ovalbumin into stimulation by conjugating ovalbumin to cholera toxin or its B subunit. *Vaccine* 12:521.
- 25 Dudler, T., Machado, D. C., Kolbe, L., Annand, R. R., Rhodes, N., Gelb, M. H., Koelsch, K., Suter, M. and Helm, B. A. 1995. A link between catalytic activity, IgE-independent mast cell activation and allergenicity of bee venom phospholipase A2. *J. Immunol.* 155:2605.
- 26 Machado, D. C., Horton, D., Harrop, R. and Peachell, P. T. 1996. Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with allergen-specific IgE. *Eur. J. Immunol.* 26:2972.
- 27 Schulz, O., Sewell, H. and Shakib, F. 1998. Proteolytic cleavage of CD25, the  $\alpha$  subunit of the human T cell Interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J. Exp. Med.* 187:272.
- 28 Vrtala, S., Hirtenlehner, K., Vangelista, L., Pastore, A., Eichler, H., Sperr, R., Valent, P., Ebner, C., Kraft, D. and Valenta, R. 1997. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments. candidates for a novel form of specific immunotherapy. *J. Clin. Invest.* 99:1673.
- 29 Elsayed, S. and Stavseng, L. 1994. Epitope mapping of region 11-70 of ovalbumin (Gal d I) using five synthetic peptides. *Int. Arch. Allergy Immunol.* 104:65.