

Allergen-specific immunosuppression by mucosal treatment with recombinant Ves v 5, a major allergen of *Vespula vulgaris* venom, in a murine model of wasp venom allergy

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SUMMARY

Up to 5% of the population suffer from systemic, 19% from local allergic hypersensitivity reactions to stinging insects. Even though specific immunotherapy is very effective in treating allergy to insect venom, new concepts of treatment strategies with only the disease eliciting allergen in recombinant form, along with antigen application via a less invasive route might be suggested for enhanced treatment efficacy and compliance. In the present study we aimed (i) to establish a mouse model of wasp venom allergy, mimicking the natural mode of sensitization, and (ii) to develop a prophylactic treatment strategy based on mucosal tolerance induction, using one major wasp venom allergen in recombinant form, i.e. recombinant (r)Ves v 5. Immunization with wasp venom – with or without the use of the adjuvant aluminium hydroxide – led to comparable T helper 2-like immune responses *in vivo* and *in vitro*. Intranasal administration of rVes v 5 prior to sensitization with wasp venom resulted in a significant reduction of wasp venom-specific antibody levels (immunoglobulin E (IgE)/IgG2a), type I hypersensitivity reactions *in vivo* and cytokine production *in vitro*. Pretreatment with the whole venom was less effective and caused toxic side reactions in higher concentrations, suggesting a favourable use of the recombinant venom allergen for mucosal application. Increased mRNA levels of transforming growth factor- β and interleukin-10, along with adoptive cell transfer experiments indicated that the immunosuppression after intranasal rVes v 5-application has been mediated by regulatory mechanisms. This is further supported by the fact that the immunosuppression to rVes v 5 was associated with a bystander suppression to the unrelated aero-allergen Bet v 1. In conclusion, we demonstrated that the intranasal application of recombinant Ves v 5 prevented subsequent allergic sensitization to all components of the whole wasp venom. As allergy to insect venom develops in dependence of the frequency of insect stings, a prophylactic treatment based on mucosal tolerance induction with recombinant allergens might be of interest for people at high risk to frequent exposure to the stinging insects.

INTRODUCTION

Stinging insects are members of the order Hymenoptera of the class Insecta. In Europe there are two major subgroups: vespids (Vespidae) including the common wasps (*Vespula vulgaris*, *Vespula germanica*), hornets (*Vespa crabro*) and paper wasps (*Polistes annularis*);^{1,2} and bees (Apidae), including the honeybee (*Apis mellifera*) and the bumblebee (*Bombus*). Hymenoptera venom is composed of biogenic amines, basic peptides and higher molecular weight proteins, mostly enzymes. Because of the protein hyaluronidase, which is contained in both honeybee and vespid venom, an immunological cross-reactivity between

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Abbreviations: c.p.m., counts per minute; H₂O₂, hydrogen peroxide; IFN- γ , interferon- γ ; IL, interleukin; n.s., not significant; TGF- β , transforming growth factor- β ; Th, T helper.

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the two venom subgroups has been described.³ The clinical features of wasp venom allergy are widespread, from painful local swelling to systemic anaphylactic reactions and shock symptoms. With one sting up to 17 µg of the vespids venom can be injected into the skin, which can be sufficient for sensitization in man.⁴

Three allergens of *Vespula vulgaris* venom, the phospholipase A₁ (Ves v 1), the hyaluronidase (Ves v 2) and the antigen 5 (Ves v 5) have been identified and sequenced.⁵ Homologues of these major allergens are present in other vespid venoms, explaining their immunological cross-reactivity.^{6,7} Ves v 5 is a 23 000 MW protein with unknown biologic function, which constitutes about 1.7% of the complete *Vespula vulgaris* venom.⁸ Ves v 5 has been sequenced,⁹ expressed in recombinant form in bacteria and yeast¹⁰ and has been structurally characterized.¹¹

Today immunotherapy (i.e. subcutaneous injection of increasing doses of allergen preparations) is the only curative treatment against allergies, and beneficial effects can persist for many years.^{12,13} Vaccines used for immunotherapy are based on extracts derived from natural allergen sources.¹⁴ The production of such vaccines, in particular of vespid venom, is laborious and cost intensive, as it is difficult to obtain large amounts of natural allergen preparations with homogeneous/standardized allergen composition.^{1,3,15} Moreover, not all patients are allergic to the whole panel of allergens presented in the vaccine, which might cause a risk of sensitization to other allergens within the preparations used for immunotherapy. Also anaphylactic side reactions might be a particular problem during venom immunotherapy, as insect venoms contain – besides the allergens – a variety of pharmacologically active amines and enzymes that can trigger immunoglobulin E (IgE)-independent mast cell mediator release.^{16–18} Vaccines based on recombinant allergens may overcome some of these problems and offer a way of patient-tailored treatment.^{19,20}

Improvements of allergy-vaccination may also include the change of the conventional parenteral route to allergen-application via the mucosa, as such an intervention might enhance the compliance of the patients. Experimentally it is well documented that mucosal antigen administration, leading to systemic/peripheral tolerance, is a promising way to treat diseases based on immunological hyperresponsiveness.^{21–24} In this respect we have previously described in a murine model of birch pollen allergy, that mucosal application of the recombinant major allergen of birch pollen can prevent allergic sensitization, but also reduce ongoing allergic immune responses.^{25,26}

The present study was performed to establish a mouse model of sensitization to wasp venom mimicking the natural way of sensitization in man. Using this model we compared the effects of one recombinant venom allergen – rVes v 5 – with the whole venom preparation in terms of tolerance induction via the mucosa to prevent subsequent systemic allergic sensitization to *Vespula vulgaris* venom.

MATERIALS AND METHODS

Animals

Female, 6-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 Education, Science and Culture.

Antigens

Ves v 5 was expressed in *Pichia pastoris* as a secreted protein.¹⁰ Recombinant (r)Ves v 5 was purified from culture supernatants by chromatography on an SE53 resin in 20 mM NaOAc (sodium acetate), pH 4–8 and eluted with a gradient of salt, 0–0.8 M NaCl. The elutant was then concentrated with 0.3 M ammonium sulphate and fractionated on a Phenyl Sepharose column. The Phenyl Sepharose eluting was further separated on a gel filtration column.

Vespa Laboratories (Spring Mills, PA) kindly provided wasp venom.

Recombinant (r)Bet v 1, the major birch pollen allergen, was obtained from Biomay GmbH (Linz, Austria).^{25,26} Birch pollen (Allergon, Sweden) was used for the preparation of the birch pollen extract, as previously described.²⁶

Experimental design

Characterization of allergen-specific immune responses to wasp venom. In order to characterize the immune responses to wasp venom, mice ($n = 5/\text{group}$) were intraperitoneally (i.p.) injected five times with different concentrations of the wasp venom (5, 15 or 30 µg at 10-day intervals) with or without Al(OH)₃ as adjuvant. Seven days after each immunization mice were bled and the kinetics of humoral immune responses were monitored. Seven days after the last sensitization, type I skin tests were performed and spleens were taken.

Mucosal pretreatment with rVes v 5 and wasp venom. For induction of mucosal tolerance, mice ($n = 5/\text{group}$) were intranasally (i.n.) pretreated three times in weekly intervals with different concentrations of rVes v 5 (1, 10 or 30 µg), wasp venom (1, 10 or 30 µg) or with sodium chloride (NaCl) as sham-pretreatment. One week after i.n. pretreatment mice were sensitized to wasp venom (5 µg/Al(OH)₃, 3 × i.p.) as described above.

Adoptive cell transfer. To investigate whether rVes v 5-induced tolerance was transferable, pooled splenocytes ($1 \times 10^7/\text{mouse}$) from i.n. pretreated (1 µg rVes v 5, 3 × i.n.) or sham-pretreated (NaCl) animals ($n = 5/\text{group}$) were intravenously injected into naive recipients ($n = 5/\text{group}$). Four hours after the cell transfer, mice were immunized with wasp venom (5 µg/Al(OH)₃, 3 × i.p.).

Bystander tolerance. In order to investigate whether tolerance induction with rVes v 5 was associated with a bystander suppression to an unrelated antigen, mice ($n = 5/\text{group}$) were i.n. pretreated with rVes v 5 (1 µg, 3 × i.n.) or sham-pretreated with NaCl, and thereafter i.p. immunized with wasp venom and rBet v 1, the major birch pollen allergen (5 µg wasp venom + 1 µg rBet v 1/Al(OH)₃, 3 × i.p.).

Seven days after the last i.p. injection, all mice were bled, killed and the sampling was completed.

Allergen-specific serum antibodies

Allergen-specific antibody levels were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.²⁵ Briefly, microtitre plates (Nunc, Maxisorp,

Roskilde, Denmark) were coated either with 10 µg/ml wasp venom or with 2 µg/ml rBet v 1 in carbonate buffer (pH 9.6). Serum samples were diluted 1 : 1000 for IgG1, 1 : 500 for IgG2a, and 1 : 10 for IgE antibody measurement. Rat anti-mouse antibodies (IgG1, IgG2a, IgE) were used at a concentration of 1 µg/ml (Pharmingen, San Diego, CA) and thereafter peroxidase-conjugated mouse antirat IgG antibodies (1 : 2000; Jackson Immuno Laboratories Inc., West Grove, PA) were added. Colour development was performed with azino-bis(3-ethylbenz-thiazoline)-6-sulphonic acid substrate (1 mg/ml; Sigma, St Louis, MO) and 1 µl/ml 30% H₂O₂. The absorbance was measured at 405 nm/550 nm with a Dynatech micro plate reader and antibody levels were expressed as optical density (OD) values after subtraction of baseline levels from preimmune sera.

Cutaneous type I skin testing

On the day of killing, 100 µl 0.5% Evans blue (Sigma) were injected intravenously into the tail veins of the mice. Subsequently, 30 µl of wasp venom (2.5 µg/ml), rVes v 5 (2.5 µg/ml), the mast cell degranulation compound (48/80; 20 µg/ml; Sigma) and phosphate-buffered saline (PBS) as negative control were administered intradermally into the shaved abdominal skin. Twenty min later the mice were killed and the reaction was evaluated on the inverted skin as previously described.²⁵

Lymphoproliferative responses and cytokine production in spleen cell cultures

At death, spleens were taken aseptically, minced, filtered through sterile filters, and cell suspension were prepared as described.²⁷

For T-cell proliferation assays, cells were stimulated with different concentrations of wasp venom (1, 5, 15 µg/well) for 4 days in an humidified atmosphere.²⁵ The cultures were pulsed with [³H]thymidine (0.5 µCi/well; Amersham) for 16 hr and proliferative responses were measured by beta plate scintillation counting.

To measure the cytokine production *in vitro*, spleen cell suspensions were cultured in 48-well plates (Costar, Cambridge, MA) with wasp venom (5 µg/well), with rVes v 5 (25 µg/well), with birch pollen (25 µg/well) or without antigen at a concentration of 5 × 10⁶/well.²⁷ Supernatants were taken 36 hr after stimulation with wasp venom/rVes v 5, 48 hr after stimulation with birch pollen, and stored at -20° until analysis. Levels of interleukin (IL)-4, IL-5 and IL-10 were measured by mouse ELISA kits (sensitivity <5 pg/ml; Endogen, Woburn, MA). Interferon-γ (IFN-γ) levels were determined as previously described (sensitivity <15 pg/ml).²⁶ Results reflect cytokine levels in pg/ml after subtraction of baseline levels of non-stimulated cultures.

IL-10 and transforming growth factor-β (TGF-β) mRNA expression

For quantification of IL-10 and TGF-β mRNA expression, total RNA was isolated from pooled spleen cell suspensions at a concentration of 5 × 10⁶ cells as previously described.²⁵ Total RNA was isolated using RNeasy Minikit (Quiagen, Valencia, CA) according to the manufacturer's instructions, treated with DNase (Quiagen) and then reverse transcribed (RT) into cDNA

using oligo dT primers (GeneAmp reverse transcriptase-polymerase chain reaction (RT-PCR) kit; PE, Boston, MA). The DNA sequences of the primers were as follows: 5'-TGF-β: ACC GCA ACA ACG CCA TCT AT; 3' TGF-β: GTA ACG CCA GGA ATT GTT GC. The IL-10 primer pair was purchased from Biosource (Camarillo, CA). Semiquantification of the RT-PCR results was achieved by amplification of β-actin using the following DNA sequences: 5' β-actin: TGG AAT CCT GTG GCATCC ATG AAA C; 3' β-actin: TAA AAC GCA GTC CAG TAA CAG TCC G. Each RT-PCR sample was electrophoresed through a 1.2% agarose gel with ethidium bromide and scanned by a transilluminator and an image analyser system (ChemImager 4400, AlphaEaseFC; Biozym, Hessisch Oldendorf, Germany). Bands were assigned to integrated density values (IDV) and normalized to β-actin IDV.

Neutralization of the IL-10 receptor in vitro

Pooled splenocytes (5 × 10⁶/well) were cultured with rVes v 5 (25 µg/well) or without antigen in the presence of rat anti-mouse IL-10 receptor monoclonal antibodies (60 µg/ml; BD Biosciences, Heidelberg, Germany) for 36 hr.²⁸ As a control the spleen cells were cultured with or without purified rat IgG1,κ isotype antibody (BD). Cytokines were measured in the supernatants as described above, and compared with those of cell cultures with or without the respective isotype control antibody.

Statistics

The Mann-Whitney *U*-test was used for statistical analysis.

RESULTS

Characterization of allergen-specific immune responses to wasp venom

Mice were bled 1 week after each immunization and the kinetics of humoral immune responses were monitored (Fig. 1a). Seven days after the last i.p. injection, type I skin tests were performed, spleens were taken and cells were cultured with wasp venom to measure cellular immune responses *in vitro* (Fig. 1b, Table 1).

Humoral immune responses (Fig. 1a). Allergen-specific serum antibodies were detected by ELISA, showing a continuous increase of wasp venom-specific IgG1 and IgE antibodies after each i.p. immunization with wasp venom, with and without the use of Al(OH)₃. The antibody levels were comparable after injection of 5 µg, 15 µg and 30 µg venom in adjuvant- and in non-adjuvant-immunized mice (Fig. 1a). In contrast to the high IgG1/IgE antibody levels, wasp venom-specific IgG2a titres were low in all adjuvant-immunized mice: 0.263 (= OD post 5 × 5 µg venom i.p.), 0.354 (= OD post 5 × 15 µg venom i.p.), 0.369 (= OD post 5 × 30 µg venom i.p.). Similarly, in mice immunized with wasp venom without Al(OH)₃, specific IgG2a antibody levels were low (0.115 = OD post 5 × 15 µg venom i.p.), or not detectable (post 5 × 5 µg and 5 × 30 µg venom i.p.).

In accordance with the high IgG1/IgE antibody levels measured by ELISA, the *in vivo* type I skin reactions were positive among intradermal challenge with the venom as well as

Table 1. Lymphoproliferation in spleen cell cultures of wasp venom-immunized mice

Immunization	Medium	1 µg/well	5 µg/well	15 µg/well
5 µg venom i.p.	10 402 ± 2861	45 669 ± 9417	35 862 ± 5450	27 918 ± 7466
15 µg venom i.p.	8797 ± 3495	38 247 ± 4604	29 391 ± 4166	20 813 ± 3779
30 µg venom i.p.	15 664 ± 2787	36 427 ± 5627	28 237 ± 3171	19 773 ± 2993

For characterization of immune responses to wasp venom, mice ($n = 5/\text{group}$) were i.p. immunized five times with different concentrations of wasp venom (5, 15, 30 µg venom i.p.). Seven days after the last i.p. injection, mice were killed and proliferation of spleen cells was measured after *in vitro* culture with increasing antigen doses (1, 5, 15 µg venom/well) and medium alone for four days. The results are shown as means of [^3H]thymidine uptake (c.p.m.) of triplicate cultures ± SD.

with rVes v 5. Both sensitization schemes – with and without adjuvant – resulted in comparable strong type I skin reactions.

Lymphocyte proliferation (Table 1). Strong proliferative responses were observed in spleen cell cultures of all wasp venom-immunized mice. Increasing doses of wasp venom used for *in vitro* stimulation exhibited cytotoxic effects, since cell viability in the cultures was decreased in a dose-dependent manner, resulting in a marked reduction of proliferative responses (Table 1). Toxicity of the venom was also observed after *in vivo* application (i.p. or i.n.), as the majority of mice died during the high-dose (i.e. 30 µg) treatment.

Cytokine production (Fig. 1b). Cytokine levels measured in spleen cell cultures of all groups displayed a T helper 2 (Th2)-pronounced cytokine profile, as indicated by high IL-4, IL-5 vs. low IFN-γ levels (Fig. 1b). IL-4 and IL-5 levels were comparable between mice immunized with and without adjuvant, whereas IFN-γ production was about 10 times lower in the non-adjuvant- than in the adjuvant-immunized animals. As already observed in the T-cell proliferation assays, sensitization with high venom concentrations/ $\text{Al}(\text{OH})_3$ had cytotoxic effects, since IL-4, IL-5 and IFN-γ production declined gradually with increasing immunization doses (Fig. 1b).

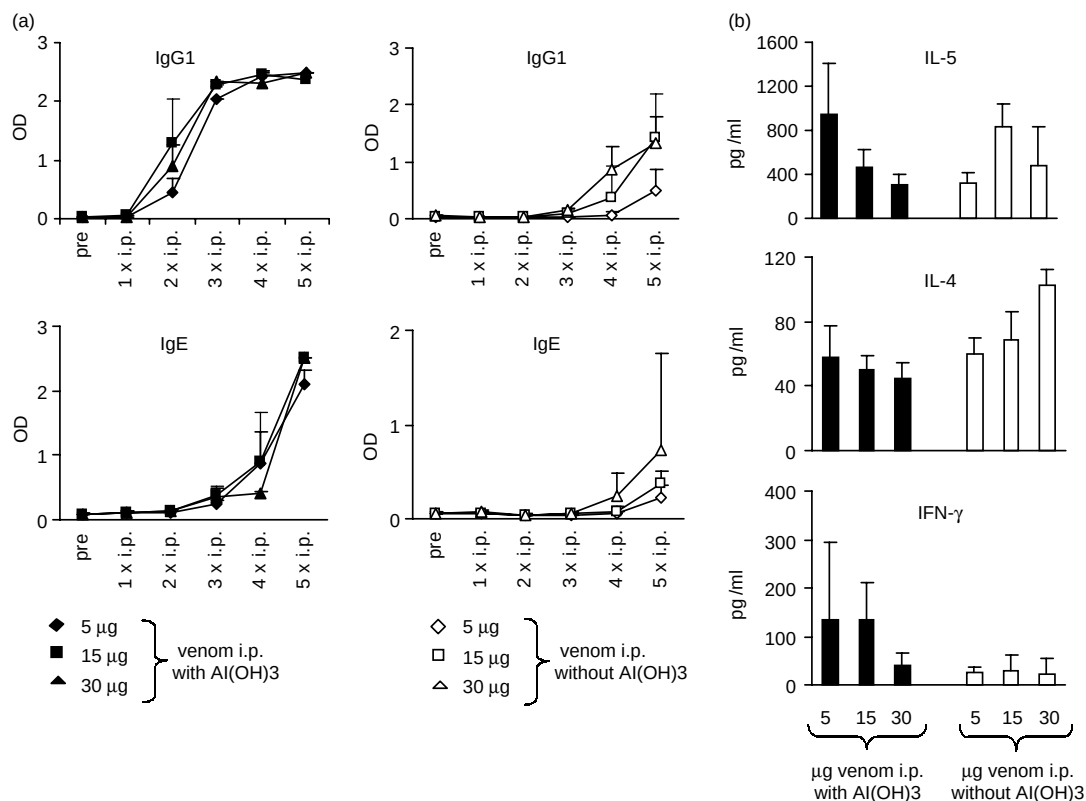


Figure 1. Allergen-specific serum antibody levels and cytokine production in spleen cell cultures of mice immunized with wasp venom with and without $\text{Al}(\text{OH})_3$. To characterize allergen-specific immune responses to wasp venom, BALB/c mice ($n = 5/\text{group}$) were intraperitoneally injected with different concentrations of wasp venom (5, 15, 30 µg venom i.p.) with (black symbols, bars) or without (white symbols, bars) $\text{Al}(\text{OH})_3$ as adjuvant. (a) Wasp venom-specific serum IgG1 and IgE levels were measured before (pre) and 7 days after each injection (1 × i.p.–5 × i.p.). Antibody levels were detected by ELISA and are reported as mean optical density (OD) values from five mice per group. (b) Seven days after the last immunization, spleen cell suspensions were prepared and cytokine levels were measured in supernatants of cell cultures 36 hr after *in vitro* re-stimulation with wasp venom. Values represent the means of five individual cytokine levels (pg/ml) per group after subtraction of baseline values. Error bars indicate standard deviations.

Mucosal pretreatment with rVes v 5 and wasp venom

For tolerization, mice were i.n. pretreated with different concentrations of rVes v 5 (1, 10, 30 µg), wasp venom (1, 10 µg) or sham-pretreated with NaCl prior to sensitization with wasp venom/Al(OH)₃. At the end of the experiment, wasp venom-specific serum antibody levels were measured by ELISA (Fig. 2a) and type I skin tests were performed (Fig. 2b). Spleen cell cultures were prepared and re-stimulated *in vitro* either with rVes v 5 (Fig. 3) or with wasp venom (Table 2).

Humoral immune responses (Fig. 2). Intranasal pretreatment with all doses of rVes v 5 was highly effective in suppressing wasp venom-specific IgE and IgG2a antibody responses. IgE antibody levels were three times lower, IgG2a production was completely abolished in the rVes v 5-pretreated compared to the sham-pretreated animals. Allergen-specific IgG1 antibody levels were slightly reduced after i.n. pretreatment with 10 µg, but remained unchanged after pretreatment with other doses of rVes v 5 (Fig. 2a).

Using the complete venom preparation for mucosal pretreatment, only the dosage of 10 µg was capable of reducing venom-specific IgE and IgG2a antibody responses. Low-dose pretreatment with the venom (i.e. 1 µg) led to enhanced IgE and unchanged IgG2a antibody levels in comparison to the sham-pretreated controls. In terms of IgG1, both treat-

Table 2. Cytokine production after *in vitro* restimulation with wasp venom in spleen cell cultures of sensitized and i.n. pretreated mice

Pretreatment	IL-4	IL-5	IL-10	IFN-γ
NaCl i.n.	34.7 ± 2	89.2 ± 26	473.2 ± 120	12.8 ± 14
1 µg rVes v 5 i.n.	20.6 ± 7*	24.3 ± 19*	194.0 ± 47*	3.6 ± 3
10 µg rVes v 5 i.n.	30.8 ± 7	34.1 ± 8*	210.5 ± 60*	53.7 ± 86
30 µg rVes v 5 i.n.	36.6 ± 13	35.8 ± 26	244.7 ± 79	14.1 ± 8
1 µg venom i.n.	30.7 ± 15	51.0 ± 42	318.5 ± 68	31.7 ± 24
10 µg venom i.n.	52.3 ± 4*	83.0 ± 8	421.8 ± 52	6.4 ± 5

For induction of mucosal tolerance, mice ($n = 5/\text{group}$) were i.n. pretreated with different concentrations of rVes v 5 (1, 10, 30 µg i.n.) or sham-pretreated with NaCl i.n. prior to sensitization with wasp venom/Al(OH)₃. Seven days after the last immunization with wasp venom/Al(OH)₃, spleens were taken and cells were *in vitro* re-stimulated with wasp venom for 36 hr, and cytokine levels were measured by ELISA. Values are shown as means of individual cytokine production from five mice per group and are expressed in pg/ml after subtraction of baseline values ± standard deviations. * $P < 0.05$ as determined by Mann-Whitney *U*-test.

ment-doses (1 µg and 10 µg venom) led to higher antibody production than the sham-pretreatment with NaCl (Fig. 2a). Intranasal application of 30 µg venom was lethal for all mice.

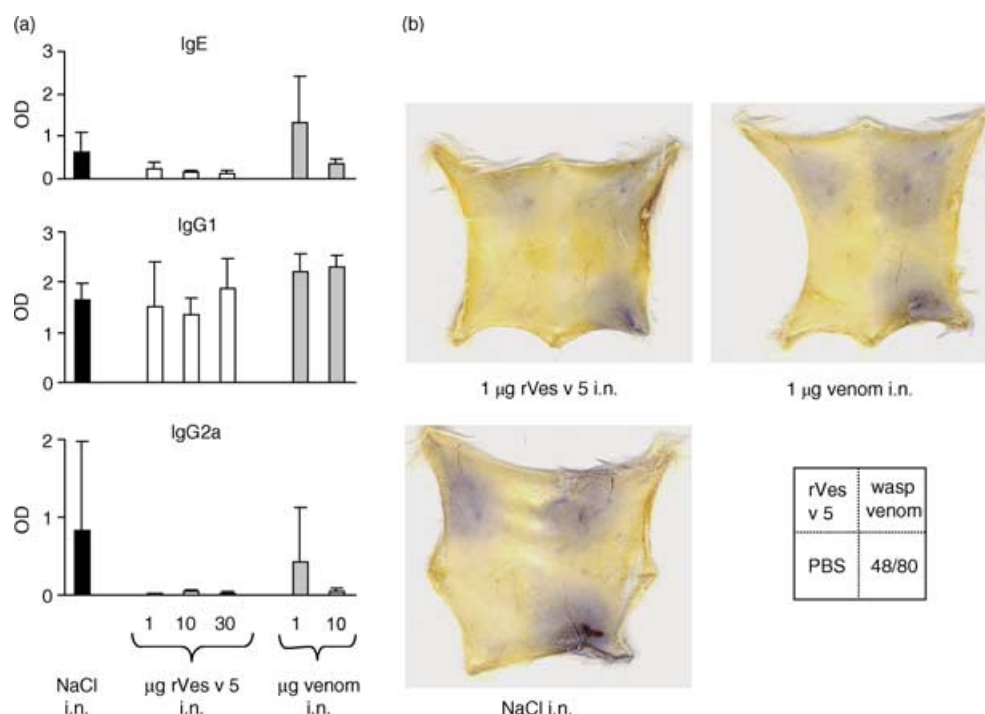


Figure 2. Wasp venom-specific serum antibody levels and type I skin tests of sensitized and i.n. pretreated mice. For induction of mucosal tolerance, mice ($n = 5/\text{group}$) were i.n. pretreated with different concentrations of rVes v 5 (1, 10, 30 µg i.n./white bars), wasp venom (1, 10 µg i.n./grey bars), or sham-pretreated with NaCl (black bars), prior to sensitization with wasp venom/Al(OH)₃. (a) Wasp venom-specific serum antibody levels were measured 7 days after the last immunization by ELISA and are expressed as mean optical density (OD) values from five mice per group after subtraction of baseline levels from preimmune sera. Error bars indicate standard deviations. (b) Representative skin sections of type I skin tests of i.n. pretreated (1 µg rVes v 5 i.n., 1 µg venom i.n.), and i.n. sham-pretreated (NaCl i.n.) animals prior to sensitization with wasp venom/Al(OH)₃. Skin reactions to wasp venom (2.5 µg/ml), to rVes v 5 (2.5 µg/ml) and to PBS were measured at the inside of the abdominal skin 20 min after intradermal injection, and were compared to their individual reaction to the histamine-releasing compound 48/80 (20 µg/ml).

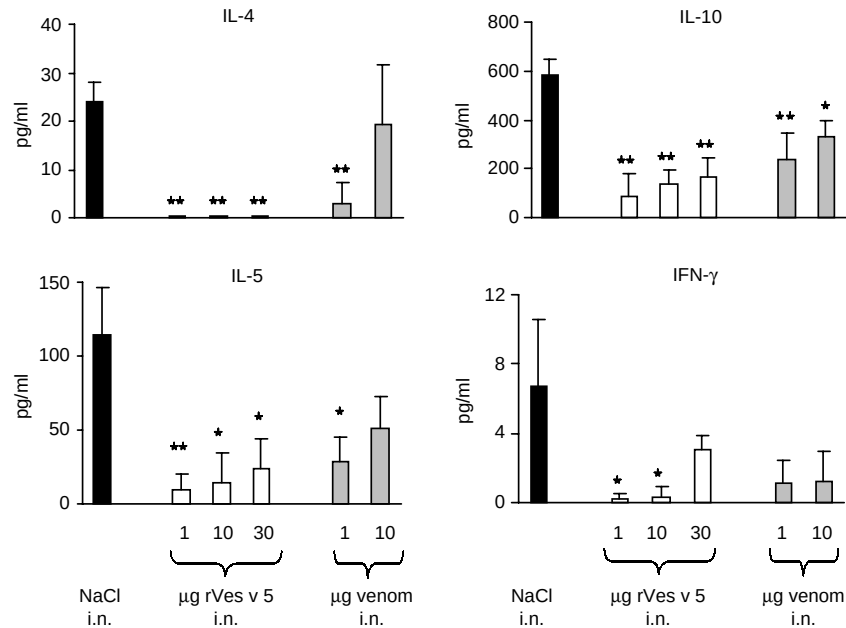


Figure 3. Cytokine production after *in vitro* re-stimulation with rVes v 5 in spleen cell cultures of sensitized and i.n. pretreated mice. Mice ($n = 5$ /group) were i.n. pretreated with different concentrations of rVes v 5 (1, 10, 30 μg i.n./white bars), wasp venom (1, 10 μg i.n./grey bars) or i.n. sham-pretreated with sodium chloride (NaCl i.n./black bars) and thereafter sensitized with wasp venom/Al(OH)₃. Seven days after the last sensitization, spleens were taken and cells *in vitro* re-stimulated with rVes v 5 for 36 hr. Cytokine levels (pg/ml) were measured by ELISA and are expressed as means from five mice per group after subtraction of baseline values. Error bars indicate standard deviations. * $P < 0.05$, ** $P < 0.01$ as determined by Mann–Whitney *U*-test.

In accordance with the decreased IgE antibody levels measured by ELISA, the immediate-type skin reactions to rVes v 5 as well as the complete wasp venom were reduced in mice i.n. pretreated with rVes v 5. In mice pretreated with wasp venom, the skin test reactivity did not differ from those of sham-pretreated control animals (Fig. 2b).

Cytokine production after *in vitro* re-stimulation with rVes v 5 (Fig. 3). Intranasal pretreatment with all doses of rVes v 5 resulted in a complete prevention of IL-4 ($P < 0.01$), and a significant reduction of IL-10 and IL-5 production ($P < 0.01$). Additionally, IFN- γ production was markedly decreased in cell cultures of mice i.n. pretreated with 1 μg and 10 μg rVes v 5 in comparison to the sham-pretreated controls ($P < 0.05$, Fig. 3).

Using the complete wasp venom for i.n. pretreatment, only the dosage of 1 μg led to a marked reduction of IL-4, IL-10 ($P < 0.01$) and IL-5 ($P < 0.05$) production. IFN- γ levels were also decreased, though not statistically significant. I.n. pretreatment with 10 μg of the venom resulted in a decrease of IL-10 production ($P < 0.05$), a moderate reduction of IL-5 and IFN- γ levels (both n.s.), whereas IL-4 levels remained unchanged compared to those of sham-pretreated controls (Fig. 3).

Cytokine production after *in vitro* re-stimulation with wasp venom (Table 2). Mice i.n. pretreated with 1 μg rVes v 5 displayed significantly lower IL-4, IL-5 and IL-10 ($P < 0.05$), and by 70% reduced IFN- γ levels than the sham-pretreated controls. Intranasal pretreatment with 10 μg rVes v 5 was capable of reducing IL-4 (n.s.), IL-5 and IL-10 production ($P < 0.05$). Pretreatment with 30 μg rVes v 5 resulted in decreased IL-5 and IL-10 (50–60%), but unchanged

IL-4 and IFN- γ levels compared to those of sham-pretreated controls (Table 2).

Concerning mucosal pretreatment with the whole wasp venom, cytokine levels were either unchanged or even significantly enhanced – in particular after i.n. pretreatment with 10 μg – compared to those of sham-pretreated animals (Table 2).

TGF- β and IL-10 mRNA expression. To study a potential role of TGF- β and IL-10 in mediating peripheral tolerance, mRNA expression was measured in pooled spleen cells from mice ($n = 5$ group) i.n. pretreated with rVes v 5, with wasp venom or sham-pretreated with NaCl and then sensitized with wasp venom/Al(OH)₃. Bands of TGF- β and IL-10 RT-PCRs were assigned as integrated density values (IDV) and normalized to β -actin IDV.

Mice i.n. pretreated with rVes v 5 and with wasp venom, displayed an increase in TGF- β mRNA expression of 52% compared to sham-pretreated animals (NaCl i.n. 1.02 IDV, rVes v 5 i.n. 1.53 IDV, wasp venom i.n. 1.56 IDV).

Regarding IL-10, mRNA expression was about 44% higher in the rVes v 5-pretreated animals than in the sham-pretreated controls. In mice i.n. pretreated with the wasp venom, IL-10 mRNA levels were reduced to 30% compared to those of i.n. sham-pretreated controls (NaCl i.n. 1.63 IDV, rVes v 5 i.n. 2.34 IDV, wasp venom i.n. 1.12 IDV).

Neutralization of the IL-10 receptor *in vitro* (Fig. 4). To study whether the decreased IL-10 levels in the cell cultures of rVes v 5-tolerized animals could be explained by an increased consumption via up-regulated IL-10 receptors, spleen cells from rVes v 5-tolerized and sham-pretreated mice prior to

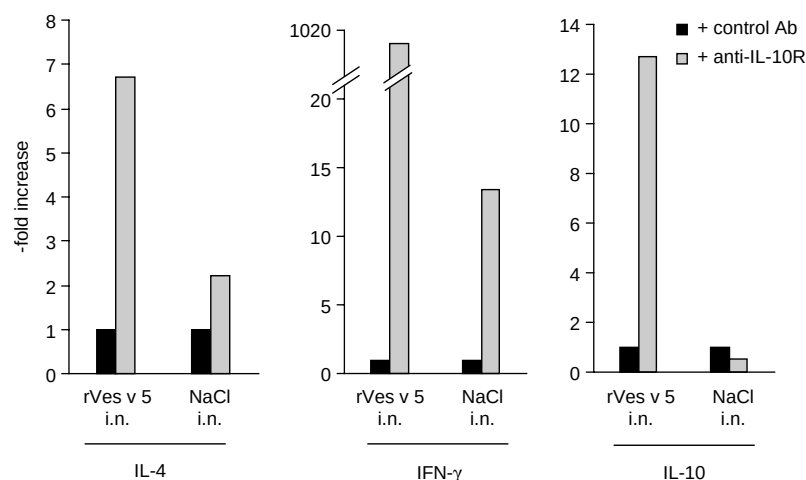


Figure 4. Neutralization of the IL-10 receptor *in vitro*. Mice ($n = 5/\text{group}$) were i.n. pretreated (rVes v 5 i.n.) or sham-pretreated (NaCl i.n.) and thereafter sensitized with wasp venom/Al(OH)₃. Pooled splenocytes were stimulated with rVes v 5 in the presence of a neutralizing anti-IL-10 receptor antibody (anti-IL-10R/grey bars) or an isotype control (control antibody/black bars). Supernatants were taken 36 hr after stimulation and cytokines were measured by ELISA. Values obtained from cultures with the anti-IL-10R antibody are expressed as -fold increase of those received after incubation with the control antibody.

sensitization with wasp venom were cultured with rVes v 5 in the presence of a neutralizing IL-10 receptor antibody (IL-10R antibody).

Addition of the neutralizing IL-10R antibody to splenocytes of rVes v 5-tolerized mice led to a sevenfold increase of IL-4, a 13-fold increase of IL-10, and a 1000-fold increase of IFN- γ levels compared with those from cultures treated with isotype control antibody. Supernatants from sham-pretreated cells also displayed elevated IL-4 and IFN- γ , but no elevated IL-10 levels in the presence of the neutralizing IL-10R antibody (Fig. 4).

No differences in cytokine production were noted between cultures treated with the control antibody or without (data not shown).

Adoptive cell transfer

To investigate whether tolerance was transferable, adoptive cell transfer experiments with spleen cells from rVes v 5-pretreated (tol) and from NaCl-sham-pretreated (control) animals were performed. Seven days after sensitization with wasp venom/Al(OH)₃, mice were bled and allergen-specific antibodies were

measured by ELISA. Cytokine production was determined in spleen cell cultures after *in vitro* re-stimulation with rVes v 5 or with wasp venom for 36 hr (Table 3).

Humoral immune responses. Recipients of rVes v 5-tolerized cells displayed slightly decreased wasp venom-specific IgG1 antibody titres (control: 1.882 ± 0.17 OD, tol: 1.709 ± 0.06 OD) and to 50% reduced IgG2a levels compared to the sham-treated animals (control: 0.276 ± 0.08 OD, tol: 0.144 ± 0.08). Specific IgE antibodies were enhanced in sera of mice transferred with rVes v 5-tolerized cells compared to the sensitized control group (control: 0.658 ± 0.48 OD, tol: 1.119 ± 0.48 OD).

Cytokine production after *in vitro* re-stimulation with rVes v 5 (Table 3). In mice receiving splenocytes from rVes v 5-tolerized animals prior to sensitization with wasp venom, IL-4, IL-5, IL-10 and IFN- γ levels were markedly reduced compared to the sensitized control mice (n.s., Table 3).

Cytokine production after *in vitro* re-stimulation with wasp venom (Table 3). Transfer of rVes v 5-tolerized cells led to significantly suppressed IL-4 production upon wasp venom re-stimulation *in vitro* ($P < 0.05$, Table 3). IL-5 levels remained

Table 3. Cytokine production of mice transferred with spleen cells from tolerized and sham-pretreated animals

Pretreatment <i>in vitro</i> stimulation	IL-4	IL-5	IL-10	IFN- γ
rVes v 5				
control	39.9 ± 9	306.8 ± 84	324.3 ± 95	31.6 ± 14
tol	34.0 ± 11	161.4 ± 56	225.4 ± 53	5.3 ± 4
wasp venom				
control	51.5 ± 6	553.4 ± 165	421.5 ± 153	60.3 ± 63
tol	$25.7 \pm 12^*$	597.9 ± 271	543.4 ± 192	39.8 ± 28

Splenocytes from mice ($n = 5/\text{group}$) i.n. treated with rVes v 5 (tol) or sham-treated with NaCl (control) were injected intravenously into naive recipients prior to sensitization with wasp venom/Al(OH)₃. Spleen cell cultures of the recipients were prepared seven days after the last sensitization and stimulated with rVes v 5 or with wasp venom for 36 hr. Cytokines were measured in the supernatants and are expressed as means from five mice per group (pg/ml) after subtraction of baseline values $\pm$ standard deviations. $*P < 0.05$ as determined by Mann-Whitney *U*-test.

unchanged, IL-10 levels were slightly increased, and IFN- γ levels were reduced by 34% compared with levels of the respective control cultures (Table 3).

Bystander tolerance

To investigate whether tolerance induction with rVes v 5 also induces bystander tolerance to an unrelated allergen, rVes v 5-pretreated or sham-pretreated animals were immunized with a mixture of wasp venom and rBet v 1/Al(OH)₃. Seven days after the last i.p. injection, allergen-specific antibody levels were measured by ELISA (Fig. 5a), and spleen cells were *in vitro* re-stimulated with birch pollen to measure the allergen-specific cytokine production (Fig. 5b).

Humoral immune responses (Fig. 5a). Bet v 1-specific IgG1 levels were significantly reduced in the rVes v 5-pretreated compared to the sham-pretreated animals ($P < 0.05$, Fig. 5a). Also Bet v 1-specific IgE antibody levels were reduced by 60% (Fig. 5a).

Cytokine production upon re-stimulation with birch pollen (Fig. 5b). rVes v 5-pretreatment led to significant reduction of IL-5 levels ($P < 0.05$) and a moderate suppression of IFN- γ production (n.s) upon re-stimulation with birch pollen compared to the sham-pretreated controls (Fig. 5b).

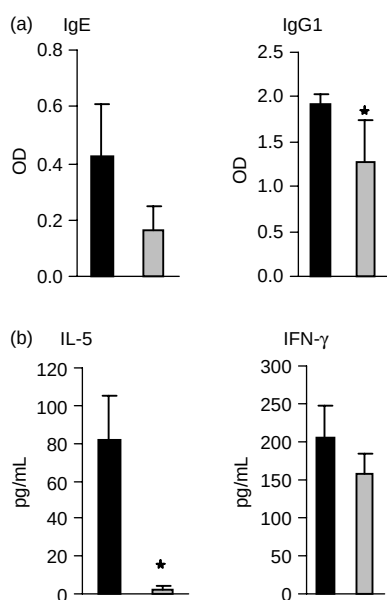


Figure 5. Bystander suppression to birch pollen after intranasal pretreatment with rVes v 5. Mice ($n = 5$ /group) were i.n. pretreated with rVes v 5 (grey bars) or sham-pretreated with NaCl (black bars) and then immunized with a mixture of wasp venom and rBet v 1/Al(OH)₃. Seven days after the last sensitization blood was taken and spleen cell cultures were prepared. (a) Bet v 1-specific antibody levels were measured by ELISA. Values are expressed as optical density (OD) means from five mice per group after subtraction of baseline levels from preimmune sera. (b) Spleen cells were stimulated *in vitro* with birch pollen for 48 hr and cytokine levels were measured by ELISA. Bars represent means (pg/mL) from five mice per group after subtraction of baseline values. Error bars indicate standard deviations. * $P < 0.05$ as determined by Mann–Whitney *U*-test.

DISCUSSION

Animal models, representing immunological characteristics comparable to those of human disease, are important tools for preclinical studies on new treatment strategies. In this respect we have established a mouse model of wasp venom allergy in order to evaluate the efficacy of tolerance induction with a recombinant venom allergen as an alternative intervention to conventional venom immunotherapy. In order to mimic sensitization to wasp venom in man, mice were systemically immunized with the natural venom preparation with and without the use of the adjuvant Al(OH)₃, known to bias Th2-like immune responses. Already after three to four systemic allergen applications without Al(OH)₃, the mice developed similar levels of allergen-specific antibodies as well as IL-4 and IL-5 levels as those mice immunized with wasp venom allergen adsorbed to Al(OH)₃. Moreover, the IgE-binding profile of mice immunized with wasp venom with or without Al(OH)₃ was comparable to that of wasp venom allergic individuals,²⁹ indicating the clinical relevance of this experimental model.

High-dose sensitization with 30 μ g of the natural wasp venom elicited severe toxic effects, as the majority of the mice died after three immunizations, and *in vitro* stimulation of splenocytes with such concentrations led to a significant decrease in cell viability. These effects might be explained by enzymatic and cytolytic actions of the allergen compounds, such as the phospholipase (Ves v 1) and the hyaluronidase (Ves v 2), since similar effects have been described for bee venom, which also contains phospholipase, different proteases and proteolytic enzymes that can trigger IgE-independent mast cell degranulation and cytotoxic damage.^{16–18} Unlike Ves v 1 and Ves v 2, no enzymatic activities have been described for Ves v 5. Accordingly, immunization with recombinant Ves v 5 gave rise to allergen-specific humoral and cellular immune responses without any toxic side effects, even after high-dose application (data not shown). Thus, in contrast to the complete venom, rVes v 5 seems to be a safe candidate for *in vivo* administration, in particular for application via the mucosal route.

With respect to the use of recombinant allergens for mucosal tolerance induction as a treatment strategy against allergic sensitization, we have previously shown in a murine model of birch pollen allergy that intranasal application of recombinant Bet v 1 (the major birch pollen allergen) led to significant suppression of allergen-specific humoral and cellular immune responses in naïve and in sensitized mice.^{25,26} Similarly, in the present study intranasal pretreatment with doses as low as 1 μ g (but also higher than 1 μ g) of rVes v 5 were effective in preventing allergic sensitization at the B- and T-cell level. More importantly, immunosuppression was not only directed against rVes v 5 itself, but towards the whole venom. Using the whole wasp venom for mucosal pretreatment, the effects were less consistent, since pretreatment with 1 μ g only suppressed cytokine production, whereas treatment with doses of 10 μ g were necessary to reduce allergen-specific IgE and IgG2a levels. The fact that intranasal pretreatment with higher doses than 10 μ g was lethal for all mice – similarly as described after systemic immunization with the venom – clearly indicates a favourable use of rVes v 5 for mucosal tolerance induction.

Mechanisms underlying peripheral tolerance are explained in dependence of the applied antigen dose, either by development of antigen-specific T-cell anergy,^{30,31} deletion,^{32,33} or regulatory cells leading to active immunosuppression or immunodeviation.^{34,35} It has been described in a murine model of tolerance induction with the bee venom phospholipase A₂, that reduction of allergen-specific IgE responses was associated with an enhancement of IgG2a antibodies and an increase in the IFN- γ /IL-4 ratio.³⁶ Similar results were obtained in studies with ovalbumin, suggesting that the down-regulation of Th2-immunity after inhaled antigen exposure was due to enhanced IFN- γ production.³⁷ In our study suppression of allergen-specific IgE, IL-4 and IL-5 was not associated with increased IgG2a/IFN- γ levels, and therefore the antigen-specific hypo/unresponsiveness cannot be explained by immune deviation, but rather by a general immunosuppression: IL-10 is regarded as a key cytokine in tolerance induction via the mucosa^{38,39} as well as via the systemic route.⁴⁰ In previous studies we demonstrated that cytokine measurements correlated well at the protein and at the mRNA levels (e.g. IL-4, IL-5, IFN- γ).²⁵ In this study we observed that IL-10 – together with TGF- β – mRNA expression was elevated in the rVes v 5-pretreated compared to the sham-pretreated mice, although IL-10 levels measured by ELISA were significantly suppressed in the tolerized animals. Therefore we investigated whether the decreased IL-10 levels could be explained by an increased consumption of the cytokine as a consequence of highly expressed receptors. Indeed, similarly as described in a human study by Jutel *et al.*⁴¹ neutralization of the IL-10 receptor abrogated the suppression of IL-4, IFN- γ and IL-10 in the cell cultures of the tolerized cells, indicating that IL-10 was one mediator of the immunosuppression in our model. Similar to IL-10, TGF- β is known as a suppressive cytokine, which, as in our mouse model of birch pollen allergy, was also increased at the mRNA level in tolerized mice.⁴² Together with a recent publication in the human system, one might argue that IL-10 and TGF- β have additive effects in the tolerizing process.⁴¹ Another indication for active suppression was the fact that tolerance was transferable via splenocytes from rVes v 5-tolerized mice, as indicated by decreased IgG1/IgG2a and IL-5 levels in the recipients after sensitization with wasp venom. Moreover, immunosuppression was even elicitable towards the unrelated allergen Bet v 1, a phenomenon known as bystander tolerance.^{43,44}

Taken together, we demonstrated that mucosal pretreatment with rVes v 5 prevented allergic sensitization to all components present in the wasp venom preparation. In contrast to the whole venom, all doses of the recombinant allergen used for intranasal pretreatment were well tolerated and did not induce any toxic side reactions. Studies on the underlying mechanisms indicated that active suppression – most likely via the production of IL-10 and TGF- β – played a central role in rVes v 5-induced tolerance.

Allergic sensitization to hymenoptera venom can occur in atopic as well as in non-atopic individuals and is correlated to the frequency of insect stings.⁴⁵ In view of the high percentage of severe systemic reactions and lethal anaphylactic reactions in venom allergic patients^{46,47} individuals with high risk to frequent exposure to stinging insects, such as workers in fruit trade, agriculture and horticulture or people with frequent out-door activities⁴⁸ could be candidates for future prophylactic inter-

vention trials with recombinant wasp venom allergens based on mucosal tolerance induction.

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