

Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes

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Novel approaches for the prevention of allergy are required, because of the inevitably increasing prevalence of allergic diseases during the last 30 years. Here, a recombinant chimeric protein, which comprises the whole amino acid sequences of three bee venom major allergens has been engineered and used in prevention of bee venom sensitization in mice. Phospholipase A₂ (Api m 1), hyaluronidase (Api m 2) and melittin (Api m 3) fragments with overlapping amino acids were assembled in a different order in the Api m (1/2/3) chimeric protein, which preserved entire T cell epitopes, whereas B cell epitopes of all three allergens were abrogated. Accordingly, IgE cross-linking leading to mast cell and basophil mediator release was profoundly reduced in humans. Supporting these findings, the Api m (1/2/3) induced 100 to 1000 times less type-1 skin test reactivity in allergic patients. Treatment of mice with Api m (1/2/3) led to a significant reduction of specific IgE development towards native allergen, representing a protective vaccine effect *in vivo*. These results demonstrate a novel prototype of a preventive allergy vaccine, which preserves the entire T cell epitope repertoire, but bypasses induction of IgE against native allergen, and side effects related to mast cell/basophil IgE FcεRI cross-linking in sensitized individuals.

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Introduction

Allergic diseases affect more than 30% of the population in industrialized countries, with an increasing prevalence during the last decades [1]. A necessity for the

development of preventive allergy vaccines has occurred, however facing major challenges. It is a prerequisite that the preventive vaccine should not lead to sensitization against the native allergens instead of protection and should not cause severe side effects in already sensitized individuals. In addition, given the huge repertoire of allergens and risk for multiple sensitization, another major task has been to generate vaccines that comprise several key allergens in one protein, which may reduce production costs of the preventive vaccine and provide broader usage.

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Abbreviations: BV: bee venom · PIT: peptide immunotherapy

The immune response to bee venom (BV) provides an especially suitable model to study human cellular and molecular mechanisms regulating allergy and normal immunity [2, 3]. Phospholipase A₂ (Api m 1) and hyaluronidase (Api m 2) represent major allergens in BV [4, 5]. The cytolytic peptide melittin (Api m 3) with potent pharmacological activity is the major component in BV and is possibly responsible for many of the side effects in BV allergen immunotherapy. IgE antibodies to Api m 3 were detected in one third of the cases [6]. Allergen exposure of sensitized individuals triggers rapid mediator release from mast cells and basophils, causing immediate allergic reactions by cross-linking FcεRI-bound IgE antibodies. This early response is characteristically followed a few hours later by a late-type response with cellular infiltrates of activated T cells, eosinophils, macrophages and fewer neutrophils [7, 8].

Antibody-binding structures on allergens are dependent on intact tertiary structure; in contrast, T cells recognize linear peptides [9]. Whereas specific B cells most efficiently present conformational allergens at low concentration, APC that utilize phagocytosis or pinocytosis for antigen uptake, such as monocytes, macrophages and/or dendritic cells, internalize allergen independently of their structural features [9, 10]. Our approach in the generation of a preventive allergy vaccine was to decrease the binding efficiency of B cell epitopes, while preserving T cell epitopes [9]. Intact T cell epitopes are required in order to enable the induction of specific T cell tolerance [11]. In contrast, B cell epitopes binding to IgE are prerequisites for sensitization against the native allergen and elicitation of adverse reactions.

Based on this novel concept, we generated a recombinant chimeric vaccine, the Api m (1/2/3), consisting of Api m 1 as two fragments, Api m 2 as three fragments and Api m 3. In Api m (1/2/3), T cell epitopes that represent linear peptide sequences were preserved, whereas conformational B cell epitopes were destroyed as demonstrated by *in vitro* IgE binding and basophil degranulation and skin test reactivity in sensitized individuals. The Api m (1/2/3) chimeric protein demonstrated an efficient preventive vaccine effect in mice, protecting against the development of specific antibodies upon contact with the native allergen.

Results

Expression and characterization of the recombinant Api m (1/2/3)

The fragments of Api m (1/2/3) were produced with partially overlapping fractions to maintain the continuity of amino acid sequences in order not to exclude

any T cell epitopes (Fig. 1A). Api m (1/2/3) protein is expressed as 66-kDa His-tagged fusion protein (Fig. 1B, C). To characterize the Api m (1/2/3), we used human and mouse monoclonal antibodies (mAb) generated against Api m 1 and Api m 2. We found that mAb which recognize same epitopes as human antibodies on Api m 2, did not bind to the Api m (1/2/3). In contrast, the mAb which recognize epitopes different than humans (non-conformational), bound to the Api m (1/2/3) (Fig. 1D). In addition, we analysed the binding of human IgG4 mAb, which recognizes Api m 1 in its three-dimensional structure [12]. This mAb bound to Api m 1, but not to Api m (1/2/3), Api m 2 and BSA (Fig. 1E). These results indicate that Api m (1/2/3) did

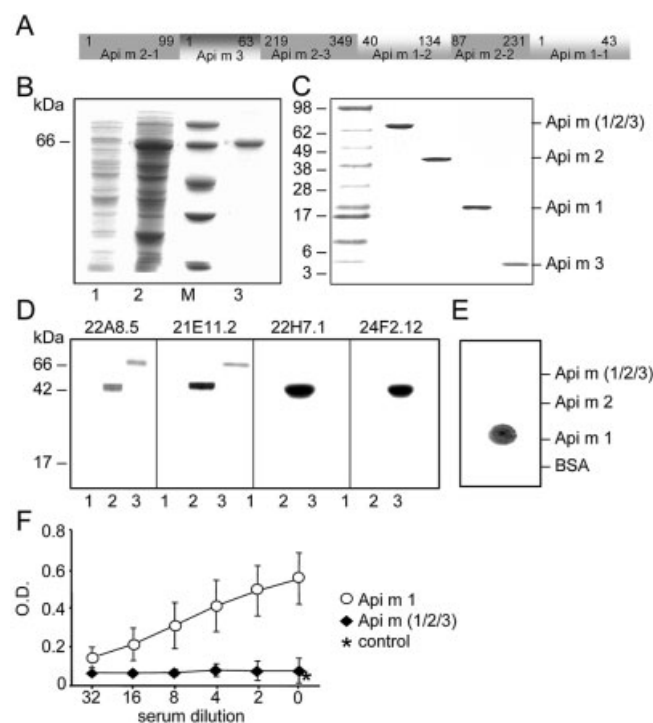


Figure 1. Characteristics of Api m 1, Api m 2, Api m 3 and Api m (1/2/3). (A) Schematic construction of Api m (1/2/3) chimeric molecule (numbering is amino acid position). (B) Induction and purification of recombinant Api m (1/2/3). Lane 1, non-induced *E. coli* carrying pET-16b; lane 2, *E. coli* after 4 h induction of protein expression; lane 3, purified Api m (1/2/3); M, molecular weight marker. (C) SDS gel electrophoresis of recombinant Api m 1, Api m 2, Api m (1/2/3) and WT Api m 3. (D–F) Api m (1/2/3) does not preserve the antibody epitopes of Api m 1 and Api m 2. (D) Binding of four different anti-Api m 2 mAb to Api m 1 (lane 1), Api m 2 (lane 2) and Api m (1/2/3) (lane 3). (E) Dot blot analysis of human anti-Api m 1 IgG4 mAb binding to Api m 1, but not to Api m 2, Api m (1/2/3) and BSA as control. (F) Api m (1/2/3) does not induce IgE against native Api m 1. Two groups of mice ($n=5$) were immunized i.p. with Al(OH)₃-adsorbed Api m 1 and Api m (1/2/3). One untreated group ($n=5$) was used as control. Api m 1-specific IgE was determined by ELISA.

not preserve the antibody epitopes of Api m 1 and Api m 2.

To support these findings in *in vivo* generated antibodies and to investigate whether there are remaining cross-reactive antibody epitopes between Api m 1 and Api m (1/2/3), mice were immunized with Al(OH)₃-adsorbed Api m 1 and Api m (1/2/3). Api m (1/2/3) did not induce any IgE response against the native Api m 1 *in vivo* (Fig. 1F).

Reduced allergenic activity of Api m (1/2/3)

Based on its characteristics above, we determined whether Api m (1/2/3) exhibits decreased binding of serum-specific IgE of BV-allergic patients. ELISA, Western blot and dot blot analysis with pooled serum from four highly sensitized BV-allergic patients showed weak to no IgE binding to the Api m (1/2/3) compared to equimolar amounts of the recombinant allergens Api m 1, Api m 2 and wild-type (WT) Api m 3 (Fig. 2A–C). The IgE binding capacity of Api m (1/2/3) was also tested in ELISA with 9 different sera of BV-allergic patients (Fig. 2D). Five of these patients showed higher IgE reactivity to Api m 1, two to Api m 2 and one to Api m 3. In all tested individuals the serum-specific IgE binding to the Api m (1/2/3) was very low or undetectable.

This was confirmed by experiments that analysed inhibition of IgE binding to solid-phase-coated allergens by soluble Api m (1/2/3) (Fig. 2E–G). The binding of patient's serum IgE to Api m 1 coated on solid phase was completely inhibited by increasing amounts of Api m 1 added to the fluid phase (Fig. 2E). In contrast, the Api m (1/2/3) added in equimolar amounts did not inhibit IgE binding to Api m 1, similar to control protein BSA. The same results were observed using Api m 2 and Api m 3 coated on solid phase (Fig. 2F, G).

The *in vitro* allergenic activity was investigated by exposing basophils from three BV-sensitized patients to equimolar concentrations of Api m (1/2/3), Api m 1, Api m 2 and BV. In all cases, the Api m (1/2/3) showed a significantly reduced or no release of basophil sulfido-leukotrienes and histamine (Fig. 3). Low allergenicity of Api m (1/2/3) was demonstrated in type-1 hypersensitivity skin-prick tests in 13 BV-allergic patients and five healthy controls. Api m (1/2/3) showed a strongly reduced to no skin reactivity compared to BV, Api m 1 and Api m 3 (Table 1).

Api m (1/2/3) retains the ability to stimulate human T cells

To investigate whether the Api m (1/2/3) is able to target specific T cells, the allergen-induced proliferative and cytokine responses in PBMC from BV-allergic

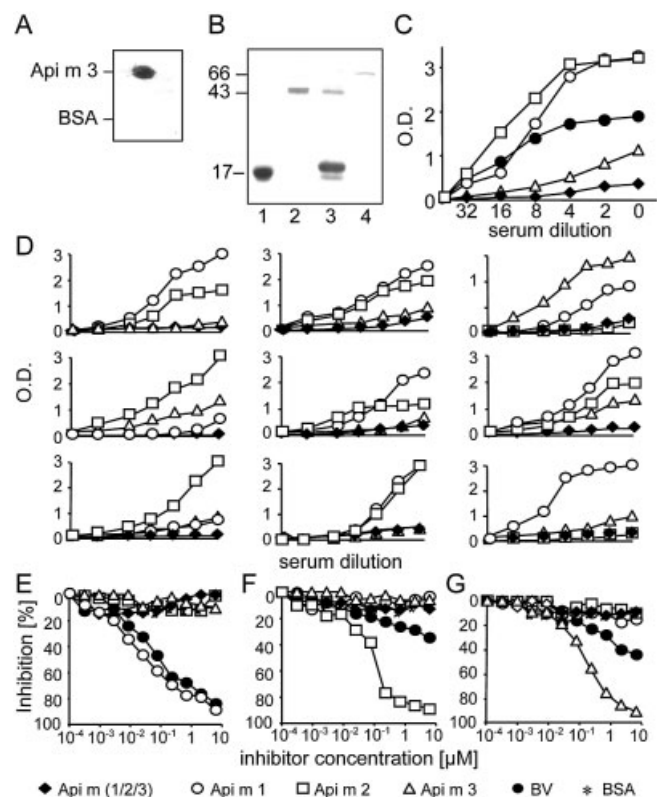


Figure 2. Api m (1/2/3) has strongly reduced BV-specific IgE reactivity. (A–C) BV-allergic patients' serum IgE binding to Api m 1, Api m 2, Api m 3, BV and Api m (1/2/3) was analysed by dot blot (A), Western blot (B): Api m 1 (lane 1), Api m 2 (lane 2), BV (lane 3) and Api m (1/2/3) (lane 4), and (C) ELISA. (D) Serum IgE binding from nine different BV-allergic patients to equimolar amounts of Api m 1, Api m 2, Api m 3 and Api m (1/2/3). Api m (1/2/3) does not inhibit IgE binding to Api m 1 (E), Api m 2 (F) or Api m 3 (G) coated on a solid phase. BSA was used as control. Results represent one of three independently performed experiments. Pooled serum obtained

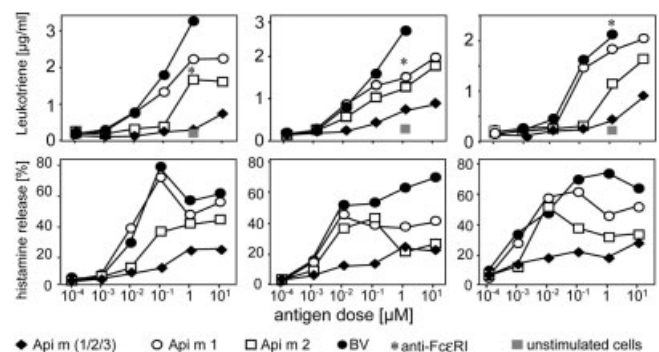


Figure 3. Reduced allergenic activity of the Api m (1/2/3). Induction of sulfido-leukotriene and histamine release from basophils of three BV-allergic patients stimulated with equimolar amounts of Api m 1, Api m 2, Api m (1/2/3) and BV. An anti-FcεRI antibody and unstimulated cells were used as positive and negative control, respectively. Results are expressed as mean values of triplicates, and represent the percentage of total histamine.

Table 1. Skin prick tests^{a)}

	Age (years)	CAP (kU/L)	BV (mm)	Api m 1 (mm)	Api m (1/2/3) (mm)	Histamine (mm)	NaCl (mm)
patients							
n=13							
mean ± SD	38.1 ± 16.3	8.3 ± 7.4	5.6 ± 1.4	3.4 ± 1.3	0.8 ± 0.7	4.2 ± 0.9	0 ± 0.0
range	21–74	1.3–26.5	3–7	0–6	0–2	3–6	
healthy							
n=5							
mean ± SD	40.60 ± 15.52	<0.35	0 ± 0	0 ± 0	0 ± 0	3.4 ± 2.3	0 ± 0

^{a)} Skin prick tests for Api m 1, BV and Api m (1/2/3) were performed in 13 BV-allergic patients and five healthy non-allergic individuals. Sodium chloride and histamine were used as negative and positive control, respectively. Mean diameter of wheal, standard deviation (SD) and range is shown; * $p < 0.001$ in comparison to Api m 1 and BV.

patients and healthy controls were analysed. As shown in Fig. 4, equimolar amounts of Api m (1/2/3) stimulated the proliferation of PBMC from BV-sensitized patients as strong as the single allergens (Fig. 4A) and induced the release of IFN- γ , IL-10 and IL-13 at the same levels as detected in stimulations with single allergens (Fig. 4B–D).

In addition, proliferation of three Api m 1-specific T cell clones, two non-specific T cell clones and one Api m 2-specific T cell line was determined after

stimulation with equimolar amounts of Api m 1, Api m 2 and Api m (1/2/3) (Fig. 4E). The Api m (1/2/3) induced proliferation in both Api m 1-specific T cell clones and Api m 2-specific T cell line similar to Api m 1 and Api m 2, respectively. Control T cell clones did not show any proliferation by stimulation with any of the three proteins.

Api m (1/2/3) vaccination suppresses the generation of anti-Api m 1 specific IgE upon allergen contact

To determine the effects of Api m (1/2/3) pretreatment on the development of anti-Api m 1 specific IgE, IgG1 and IgG2a, mice were subcutaneously (s.c.) pretreated with Api m (1/2/3) and Api m 1 in saline or with saline alone as control 14, 10 and 6 days prior to intraperitoneally (i.p.) immunization with phospholipase A₂/Al(OH)₃. In contrast to control animals, the Api m (1/2/3) and Api m 1 pretreatment resulted in the inhibition of Api m 1-specific IgE, IgG1 and IgG2a (Fig. 5A). Same strong inhibition of IgE production was observed by single-dose pretreatment 7 days prior to immunization (Fig. 5B). In Api m (1/2/3)-pretreated mice the Api m 1-specific antibody production was abrogated without showing a switch between isotypes. These data demonstrate an efficient suppression of antibody responses including IgE directed to native allergen by Api m (1/2/3) as a novel vaccine.

Api m (1/2/3) treatment induces IgG2a-dominant de novo antibody production

To investigate whether Api m (1/2/3) treatment interferes with established IgE production, we sensitized mice with whole BV and then weekly injected with Api m 1 or Api m (1/2/3) in Al(OH)₃ ten times every third day. We analysed the newly generated Api m (1/2/3)-specific IgE and Api m (1/2/3)-specific IgG2a in

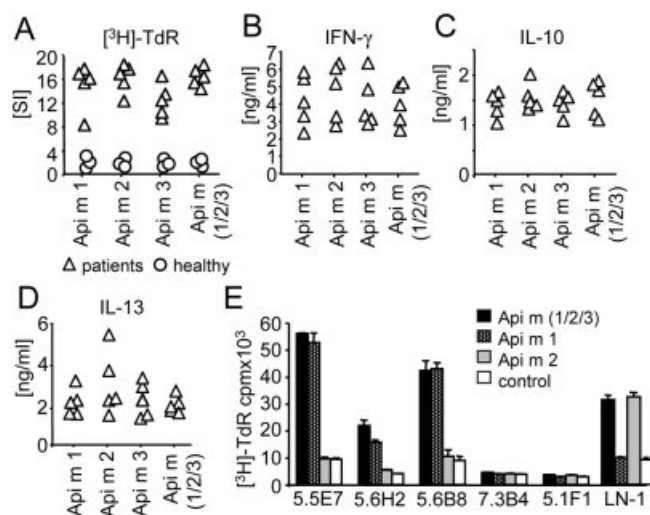


Figure 4. Api m (1/2/3) retains T cell reactivity of Api m 1, Api m 2 and Api m 3. (A) PBMC from five BV-allergic patients and three healthy controls were stimulated with 0.3- μ M amounts of Api m 1, Api m 2, Api m 3 and the Api m (1/2/3). [³H]Thymidine incorporation ([³H] TdR) was measured after 5 days. SI is stimulation index. (B–D) Supernatants were harvested after 5 days for IFN- γ , IL-10 and IL-13. (E) Api m (1/2/3) retains T cell epitopes of Api m 1 and Api m 2. Api m 1-specific T cell clones (5.5E7, 5.6H2, 5.6B8) as well as non-specific T cell clones (7.3B4 and 5.1F1) and Api m 2-specific T cell line (LN-1) were co-cultured with autologous APC and stimulated with equimolar amounts of Api m 1, Api m 2 and Api m (1/2/3). Thymidine incorporation was measured after 2 days.

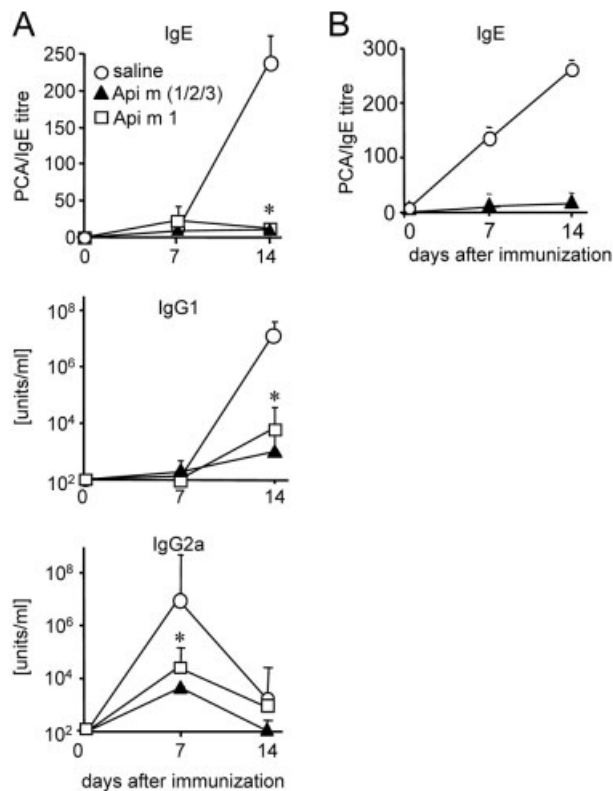


Figure 5. Api m (1/2/3) suppresses IgE, IgG1 and IgG2a development against native allergen. (A) Three groups of mice ($n=6$) were pretreated s.c. with Api m (1/2/3), Api m 1 in saline, or saline alone as control 14, 10 and 6 days prior to i.p. immunization with Api m 1/Al(OH)₃. (B) Mice were pretreated by a single dose of Api m (1/2/3) 7 days before immunization. Api m 1-specific IgE titers were measured by PCA. Api m 1-specific IgG1 and IgG2a were measured by ELISA. Error bars indicate standard deviation; * $p<0.001$.

serum samples (Fig. 6). Non-sensitized control mice did not develop any antibodies (not shown). Mice which were sensitized with BV and only received saline Al(OH)₃ showed very low or undetectable levels of Api m (1/2/3)-specific IgE. In contrast, sensitized mice, which received Api m 1 or Api m (1/2/3), both developed IgE against Api m (1/2/3). Interestingly, mice treated with Api m (1/2/3) generated high levels of Api m (1/2/3)-specific IgG2a. Accordingly, the Api m (1/2/3)-specific IgE/IgG2a ratio was significantly higher in mice which received Api m 1 injections, because of relatively high levels of Api m (1/2/3)-specific IgG2a in Api m (1/2/3)-administered mice.

Discussion

The present study demonstrates a novel preventive allergy vaccine with profoundly reduced activity for the binding, cross-linking and induction of IgE, but retained T cell reactivity. This was achieved by destruction of

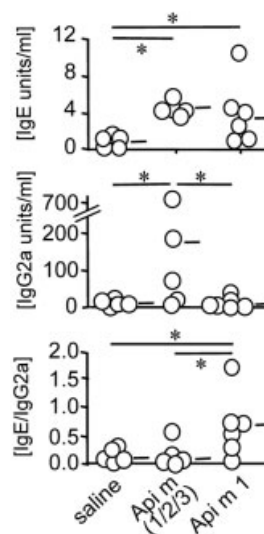


Figure 6. Api m (1/2/3) treatment induces IgG2a-dominant *de novo* anti-Api m (1/2/3) antibodies. C57BL/6 mice were immunized i.p. with BV. Control group received s.c. injections with saline alone. After sensitization the mice were treated with s.c. injections of Api m 1 or Api m (1/2/3) in Al(OH)₃ or left untreated. Api m (1/2/3)-specific IgE and IgG2a antibodies were determined in sera. * $p<0.01$.

conformational B cell epitopes and preservation of linear T cell epitopes of BV allergens in an approach that combines three major allergens in one protein. The use of Api m (1/2/3) before the sensitization to Api m 1 abrogated the Api m 1-specific IgE, IgG1 and IgG2a responses in mice, demonstrating that this model can be used as a preventive vaccine for several allergies before the sensitization phase. There was no skewing of the immune response between antibody isotypes. All of the isotypes were significantly suppressed and IgE was fully abolished even with a single dose of pretreatment 7 days before the native allergen exposure.

There are several major disadvantages of using native allergen extracts in a preventive allergy vaccine. Induction of IgE sensitization against native allergens instead of development of a protective immune response might be a major problem with current allergen extract-based vaccines [13]. In addition, some individuals might already be sensitized against some of the allergens in the vaccine content. Accordingly, the vaccine should be formulated in such a way that it does not carry any risk of side effects due to IgE cross-linking on mast cells and basophils. The Api m (1/2/3) chimeric protein is comprised of fragments of major BV allergens Api m 1, Api m 2 and Api m 3 [4, 5]. The three-dimensional structures of BV allergens Api m 1 and Api m 2 are stabilized by intra-chain cysteine disulfide bridges in their native forms [12, 14]. To destroy the tertiary structure of these allergens, Api m 1 was produced as two and Api m 2 as three fragments. Because the

proportion of promelittin found in younger bees is greater than that of melittin in older bees, we generated Api m 3 as promelittin [15]. It was expressed without residues 21–26, which are essential for the lytic activity [16].

The initiation and maintenance of allergic reactions are strongly influenced by allergen-specific T cell responses, which not only regulate IgE production, but also affect the activation and differentiation of allergic effector cells [9]. BV major allergens contain several distinct T cell epitopes [17]. These immunogenic determinants are recognized by specific T cells of both allergic and non-allergic individuals. Moreover, the same immunogenic T cell epitopes which activate T cell responses can induce anergy in T cells [18]. Accordingly, we generated Api m (1/2/3) using fragments with overlapping sequences. In this way, the continuity of amino acid sequences was preserved and thereby T cell epitopes were protected.

Api m (1/2/3) induced proliferation and cytokine secretion of T cells from BV-allergic patients and Api m 1- and in Api m 2-specific T cell lines, suggesting that it comprises the complete repertoire of T cell epitopes of major BV allergens. Accordingly, it may be expected that the use of Api m (1/2/3) may lead to stimulation of memory Th2 responses. However, the induction of specific peripheral T cell tolerance and efficiently decreased Th2 cytokines has been demonstrated both in conventional BV immunotherapy [19] and Api m 1 peptide immunotherapy [20] as well as healthy immune response to allergens [21], which might be more relevant in the present study for the prevention of IgE as well as IgG1 and IgG2a after native allergen exposure.

There is considerable rationale for targeting T cells with synthetic peptides based on such T cell epitopes. To date, clinical trials of peptide immunotherapy (PIT) have been performed in cat and BV allergies [20, 22]. These studies showed modulation of the immune response against the whole allergen, inducing specific T cell tolerance and a decrease in the specific IgE/IgG4 ratio in BV-PIT. A potential barrier to PIT of allergy is the apparent complexity of the allergen-specific T cell response in terms of epitope usage in humans. An individual's allergen-specific T cell repertoire may contain multiple specificities, probably reflecting the recognition of multiple epitopes contained within the allergenic protein [23]. Our current approach retains all of the T cell epitopes and overcomes several problems faced in PIT such as stability of short peptides, combination of several major allergens in a single protein and cost-effectiveness.

The presence of conformational B cell epitopes proposed a novel approach to reduce IgE binding by fragmentation [24] and oligomerization of one allergen

or hybridization of several allergens [25, 26]. The majority of Ab elicited by exposure to native protein antigens recognize conformation-dependent, discontinuous epitopes [2, 12, 27], which is confirmed using mouse mAb against Api m 1 and Api m 2. The mAb, which recognized the conformational epitopes of these two molecules, did not recognize the Api m (1/2/3). In addition, reduction of IgE reactivity resulted in diminished allergenicity in basophil leukotriene- and histamine-release assays and in human skin-prick tests. It has currently been demonstrated that histamine released from mast cells and basophils during allergic reactions can modify T cell and antibody responses due to distinct expression of histamine receptors on these cells [28]. In this context, Api m (1/2/3) does not induce histamine release and may have additional beneficial activity. In addition, IgE-facilitated antigen presentation, which favors the development of a Th2-dominated cytokine pattern, does not take place [9, 29].

It has to be noted here that the present study does not involve the use of any specific adjuvants to improve the preventive effect. It is probable that use of appropriate adjuvants may increase the efficiency by specifically targeting tolerance-inducing dendritic cell subsets, and enable efficient priming of the memory T cell response before the exposure to native allergen [30, 31]. Together the present study demonstrates a novel concept to be used as a preventive vaccine. A similar strategy which targets T cells and bypasses IgE can be employed in several allergies.

Materials and Methods

Study population

Peripheral venous blood (five individuals) and serum samples (17 individuals) from BV-allergic patients and three healthy controls were used. Skin-prick tests were performed in 13 BV-allergic patients and five healthy controls. The study has been approved by the ethical committee of the University of Zürich, Zürich, Switzerland (No. 381). All patients gave oral and written informed consent. For diagnosis of BV allergy, clinical history, immediate skin reactivity to commercial BV (ALK, Hoersholm, Denmark) and serum IgE levels (Unicap-system; Pharmacia, Uppsala, Sweden) were analysed.

Expression and purification of recombinant Api m (1/2/3) protein

The generation of Api m (1/2/3) DNA was performed by fusion PCR. The Api m 1 was amplified as two fragments (Api m 1–1: nucleotides 1–130, Api m 1–2: nucleotides 118–403) and Api m 2 was amplified as three fragments (Api m 2–1: nucleotides 1–297, Api m 2–2: nucleotides 259–693, Api m 2–3: nucleotides 655–1047) with overlapping nucleotide sequences. DNA of Api m 3 was synthesized by

oligonucleotide-based fusion PCR. The purified fragments were used for fusion PCR technique to generate Api m (1/2/3) chimeric DNA in a direct way. The final sequence analyses revealed a full match to native sequences of all three proteins.

Finally, the construct was cloned in the expression plasmid pET-16b (Novagen, Bad Soden, Germany) with the His₁₀ tag fused to the N terminus. The fusion protein Api m (1/2/3) was expressed in *Escherichia coli* BL21-codon Plus-RIL-(DE3) (Stratagene, East Kew, Australia). For purification, the cells were resuspended in lysis buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 5 mM imidazole, 3 M dithiothreitol, 1% SDS), boiled at 90°C for 15 min and supernatant was alkylated (10% iodoacetamide). His-tagged Api m (1/2/3) was purified under native condition using nickel-chelate-affinity chromatography (Qiagen, Basel, Switzerland). Recombinant Api m 1 and Api m 2 were produced in *E. coli* BL21 (RIL) (Stratagene) and by baculovirus-infected insect cells, respectively [12, 32].

Specific antibody binding

Sera of BV-allergic patients were tested using equimolar amounts of Api m 1, Api m 2, Api m 3 and the Api m (1/2/3) in a standard solid-phase ELISA and CAP system (Pharmacia) [33]. Serially diluted Api m 1, Api m 2, Api m 3, Api m (1/2/3) and BSA were pre-incubated for 1 h with BV-allergic patient's serum for the inhibition of antibody binding [34]. Protein samples were separated by 4–12% bis-tris polyacrylamide NuPAGE gels (Invitrogen, Basel, Switzerland) under denaturing conditions. Gels were either stained with Coomassie brilliant blue or proteins were transferred to nitrocellulose membrane (Amersham, Dübendorf, Switzerland). For Western blot analysis, equimolar amounts (1.5 µM) of the three recombinant proteins Api m 1, Api m 2 and Api m (1/2/3) were used. The membrane was probed with the diluted (1:50) pooled serum of four BV-allergic patients and bound IgE antibodies were detected as previously described [35]. Dot blot analyses were performed under native conditions with equimolar amounts (1.5 µM) of Api m 3 and BSA (as control protein).

Api m 2-specific mouse IgG mAb were generated and tested for their binding capacity to Api m 2. mAb 22H7.1 and 24F2.12 recognize the same epitopes as human antibodies, because their binding to Api m 2 was inhibited by serum of BV-allergic individuals. mAb 22A8.5 and 21E11.2 recognized epitopes which are different from the ones recognized by human antibodies. Furthermore, the capacity of human Api m 2-specific serum to inhibit this binding was investigated. These Api m 2-specific mouse IgG mAb and human mAb against Api m 1 (BVA2) [12] were tested for binding to Api m 1, Api m 2 and Api m (1/2/3) using ELISA, Western blot or dot blot analysis.

Human T cell cultures

PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood and stimulated with titrated equimolar amounts of Api m 1, Api m 2, Api m 3 and Api m (1/2/3) as described [19, 28]. The stimulation index was calculated as the quotient of counts per minutes obtained by allergen-stimulated PBMC and

unstimulated PBMC. T cell clones specific to Api m 1 and T cell line specific to Api m 2 were derived from a hyper-immunized healthy individual as described [17]. Clone 5.5E7 recognizes an epitope spanning amino acids 94–111, 5.6B8 recognizes an epitope spanning amino acids 81–98 and 5.6H2 recognizes an epitope spanning amino acids 113–124 of Api m 1. In addition, Api m 1-non-specific 5.1F1 and 7.3B4 were used as controls. Epstein-Barr virus-transfected autologous B cells were used as APC [17]. Experiments were performed in dose and time kinetics. Optimum proliferation and cytokine production at 0.6 µM antigen doses are shown.

Quantification of cytokines

The solid-phase sandwich ELISA for IFN-γ, IL-10 and IL-13 were performed in duplicates as described [19]. The sensitivity for IFN-γ was <10 pg/mL (kindly provided by Dr. C. H. Heusser; Novartis, Basel, Switzerland). The sensitivity for IL-10 was <50 pg/mL and the detection limit for IL-13 was 100 pg/mL (PharMingen, St. Louis, MO).

Basophil mediator release

Sulphido-leukotriene release from basophils was analysed by cellular antigen stimulation test (CAST)-ELISA (Bühlmann Laboratories AG, Allschwil, Switzerland). Briefly, granulocytes were isolated from three different BV-allergic patients and incubated with equimolar concentrations of recombinant Api m 1, Api m 2 and Api m (1/2/3) and BV (ALK). An antibody directed against FcεRI and unstimulated cells were used as positive and negative controls, respectively. Leukotrienes were determined in the cell supernatant by ELISA [36]. Similarly, released histamine was measured by ELISA according to the manufacturer's instructions. Results are expressed as mean values of triplicate determinations, and represent the percentage of total histamine.

Skin-prick test

Skin-prick tests were performed in duplicates in the volar forearm with commercial BV (ALK) and equimolar amounts of Api m 1 and Api m (1/2/3). Sodium chloride (0.9%) and histamine hydrochloride (ALK) were used as negative and positive controls, respectively [37].

Induction and regulation of specific antibodies in mice

All experiments complied with the Home Office 1986 animals scientific act and were approved by King's College animal committee for review of ethics & welfare or by the Kantonales Veterinäramt, Zürich, Switzerland (No. 136/2000). Two groups of female C57BL/6 mice (4–6 wk of age, n=6) were immunized s.c. with 100 µg/100 µl of Api m (1/2/3) and Api m 1 in saline 14, 10 and 6 days prior to i.p. immunization with 100 µg/100 µl of Api m 1/Al(OH)₃. Control mice (n=6) received s.c. injections with saline alone. Sera were collected at days 0, 7 and 14 and assayed for Api m 1-specific IgE, IgG1 and IgG2a antibodies. Api m 1-specific IgE antibodies were assayed by passive cutaneous anaphylaxis (PCA) using Wistar rats (Harlan Olac, Bicester, UK) and represented as the highest

dilution of serum that produced a positive mast cell-dependent PCA reaction. Api m 1-specific IgG1 and IgG2a were monitored by ELISA [38, 39]. For IgE response assays mice (three groups/five animals each) were immunized i.p. three times every 2 wk with 0.1 µg Al(OH)₃-adsorbed Api m 1, Api m 2 and Api m (1/2/3). Api m 1-specific IgE was detected by ELISA at the sixth week.

In another set of experiments, three groups of female C57BL/6 mice (6 wk of age) were immunized six times i.p. with BV in Al(OH)₃, three times 0.5 mg/100 mL and three times 5 mg/100 µL bi-weekly. Control animals (n=6) received s.c. injections with saline alone. Mice were treated with s.c. injections of 5 mg/100 mL Api m 1 or 7 mg/100 mL Api m (1/2/3) in Al(OH)₃ ten times every third day from day 100 to day 130 or left untreated. Sera were collected at day 182 and analysed for Api m (1/2/3)-specific IgE and IgG2a antibodies. Mann-Whitney U-test and paired Student's t-test were used for statistical analysis.

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