

ORIGINAL ARTICLE

ANAPHYLAXIS

Short-term venom immunotherapy induces desensitization of FcεRI-mediated basophil response

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Keywords

basophils; high-affinity IgE receptor/FcεRI; *Hymenoptera* allergy; short-term venom immunotherapy.

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Abstract

Background: The precise immunological mechanisms for the early clinical protection of venom immunotherapy (VIT) have not yet been explained. Our aim was to evaluate whether high-affinity IgE receptor (FcεRI) and the related basophil function have a role in the induction of short-term VIT protection.

Methods: We included 60 adults and 48 children. Basophil threshold sensitivity (CD-sens) to anti-FcεRI stimulation, and FcεRI gene and cell-surface expression were assessed at the beginning and just before the first maintenance dose (MD) of 100 µg of ultra-rush VIT (day 5) and at the beginning, during buildup, and just before the first MD of 70 µg and of 100 µg of semi-rush VIT (weeks 1–2 and 5).

Results: We demonstrated a significant reduction in CD-sens to anti-FcεRI stimulation before the first MD in both ultra-rush and semi-rush VIT in all included subjects. FcεRI gene and/or cell-surface expression was decreased in 34–100% of subjects, with different dynamics between VIT protocols.

Conclusion: We found a marked desensitization of FcεRI-activated basophils after short-term VIT. This suppression, which could be highly relevant for the development of early protective mechanisms, might be also related to the changes at the level of FcεRI expression.

The clinical efficacy of venom immunotherapy (VIT) has been documented and well established. Although long-lasting allergen tolerance requires at least 3–5 years of treatment, its early protection has been confirmed already after the maintenance dose (MD) had been achieved (1,2). The early protective mechanisms that lead to unresponsiveness to the sensitizing allergen seem to develop during the buildup phase of VIT (1–3). Rush and ultra-rush venom immunotherapy protocols have proven to be as safe and efficient as conventional immunotherapy (1,2,4–6).

Despite its effectiveness, the precise immunological mechanisms for the immediate protection of VIT have not yet been explained. The proposed early markers for protective mechanisms during specific immunotherapy include increased tryptophan degradation, the rise in IL-10 levels, and IL-10-

producing cell types, and up-regulation of histamine receptor 2 (3,7–12). Studies have shown reduced histamine, sulfidoleukotriene, and cytokine release during the early course of VIT, implying that their early suppression is one of the first protective mechanisms (11,13).

The high-affinity IgE receptor (FcεRI) is a key molecule on the surface of mast cells and basophils that participates in signal transduction of IgE-mediated response, and the initial FcεRI-mediated signal strength plays a major role in regulating effector cells' deactivation (14). In recent studies, the clinical efficacy of anti-IgE treatment was demonstrated within 7 days of the initial treatment, as soon as reduction in basophil FcεRI expression had been initiated (15,16). This indicates a critical role of basophils and FcεRI in the early process of clinical desensitization. Moreover, it was recently demonstrated that the subthreshold desensitization of basophils leads to the loss of FcεRI expression (17).

Because some previous data suggest an important role of FcεRI in modulating allergic response, we hypothesized that

Abbreviations

VIT, venom immunotherapy; FcεRI, high-affinity IgE receptor; MD, maintenance dose.

FcεRI changes could also be related to the early alternations induced by VIT. For this reason, we evaluated basophil threshold sensitivity to anti-FcεRI stimulation and FcεRI gene and cell-surface expression at the beginning and just before the first MD in ultra-rush and/or semi-rush VIT.

Methods

Study population

Sixty adults (mean age 46 years; range 19–73; 34 men) with a history of systemic allergic reaction after *Hymenoptera* sting [Mueller grades IV (55%), III (37%), and II (8%)], 18 children (11 years; 4–17; 13 boys) after honeybee sting [IV (6%), III (33%), II (44%), and I (17%)], and 30 children (11 years; 5–17; 25 boys) after *Vespula* sting [IV (17%), III (53%), II (27%), and I (3%)] were included in this prospective study. In 106 subjects, the sensitization was confirmed by venom-specific IgE and/or skin tests. Only two subjects had negative standard test results, but a positive basophil activation test (18). Thirty-four adults were enrolled in ultra-rush VIT with *Vespula* and 26 with honeybee venom. Eighteen children were enrolled in ultra-rush VIT with honeybee, and 30 children were enrolled in semi-rush VIT with *Vespula* venom. One child was missed before MD of 70 µg and 4 before MD of 100 µg. The control group consisted of five healthy, nonallergic individuals (31 years; 24–41; two men).

All patients gave written informed consent for treatment with specific immunotherapy and participation in the study. The study was approved by the Slovenian National Medical Ethics Committee.

Study protocol

Blood samples of patients on ultra-rush VIT were taken before the beginning of treatment and at the end of the buildup phase, before the first MD of 100 µg administration. Blood samples of patients on semi-rush VIT were taken before the beginning of treatment, during buildup, before the first MD of 70 µg, and before the first MD of 100 µg. Blood samplings were performed just before the injection of allergen. The ultra-rush and semi-rush protocols are shown in Fig. 1. The control group was monitored on days 1 and 5.

Basophil threshold sensitivity to anti-FcεRI stimulation and CD-sens calculation

A basophil activation assay was performed on the heparinized whole blood incubated with serial dilutions of anti-FcεRI mAbs (Buhlmann Laboratories, Basel, Switzerland) from 550 to 0.55 ng/ml, at 37°C for 15 min. Degranulation was stopped by chilling on ice, after which anti-CD63, anti-CD123, and anti-HLA-DR mAb (BD Biosciences, Franklin Lakes, NJ, USA) were added and incubated for 20 min. Finally, whole-blood probes were lysed, washed, fixed, and analyzed within 2 h on a FACSCalibur flow cytometer (BD Biosciences).

Basophil sensitivity was determined as the anti-FcεRI mAbs concentration giving a 50% of maximum CD63% up-regulation. CD sensitivity (CD-sens) was calculated as the inverse value of this threshold anti-FcεRI mAb concentration multiplied by 100, as previously demonstrated (19–21). The higher value for CD-sens represents higher basophil sensitivity.

FcεRI gene expression profiles

Total RNA was isolated from whole-blood samples using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Basophils were obtained from heparinized whole blood, enriched and purified by Ficoll density centrifugation, followed by magnetic cell sorting (MACS Basophil Isolation Kit II, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by Qubit® fluorometer (Invitrogen Corporation, Carlsbad, CA, USA). Following reverse transcription, cDNA was quantified by real-time PCR (ABI PRISM 7500 Real-Time PCR System) at standard conditions using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression levels of the α -subunit of high-affinity IgE receptor (*FCER1A*) (Hs00175232_m1) were normalized against 18S rRNA endogenous control (Applied Biosystems). All measurements were taken in triplicate for each sample and time point, and the relative expression was analyzed using the $\Delta\Delta C_t$ method.

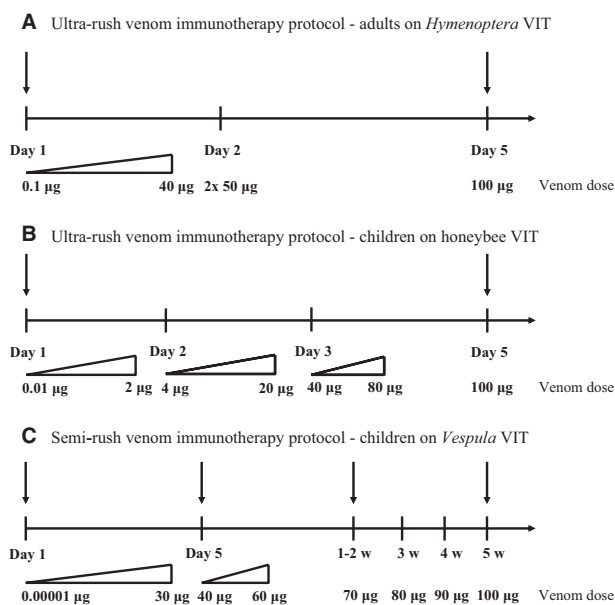


Figure 1 (A–C) Blood sampling (↓) in A 60 adults and B 18 children was performed just before ultra-rush VIT and the first MD of 100 µg and in C 30 children just before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. One child was missed before MD of 70 µg and 4 before MD of 100 µg. MD: maintenance dose

FcεRI cell-surface expression

The number of FcεRI receptors per basophil (CD123 + / HLA-DR - cells) was analyzed using a FITC-conjugated antibody to FcεRI (eBioscience, San Diego, CA, USA) and standard curve of Calibration Beads (Dako Cytomation, Glostrup, Denmark) as previously described (20).

Absolute basophil cell count

For the absolute basophil count (CD123 + / HLA-DR - cells), we added AccuCount Fluorescent microbeads (Spherotec Inc., Libertyville, IL, USA) to fixed samples before analysis. The absolute number of basophils per μl of whole blood was calculated using the following equation: (number of events for basophil region/number of events for microbead region) $\times$ (number of microbeads used in test/volume of the whole-blood sample initially used).

Statistical analyses

Data distribution was evaluated using the D'Agostino–Pearson normality test. Depending on the distribution of the data, we used either a Wilcoxon's matched pairs test or a paired *t*-test. Data were expressed as median (IQR) or mean (95% CI). Probability values (*P*) of < 0.05 were accepted as significant. Analyses were performed using GraphPad Prism 5.

Results

Basophil activation

The results of %CD63 basophil response were analyzed as dose-response curves (Fig. 2A–B) and CD-sens (Fig. 3A–B) after stimulation with serial dilutions of anti-FcεRI mAbs in 22 adults on ultra-rush VIT, in 30 children on semi-rush VIT, and in 5 healthy controls.

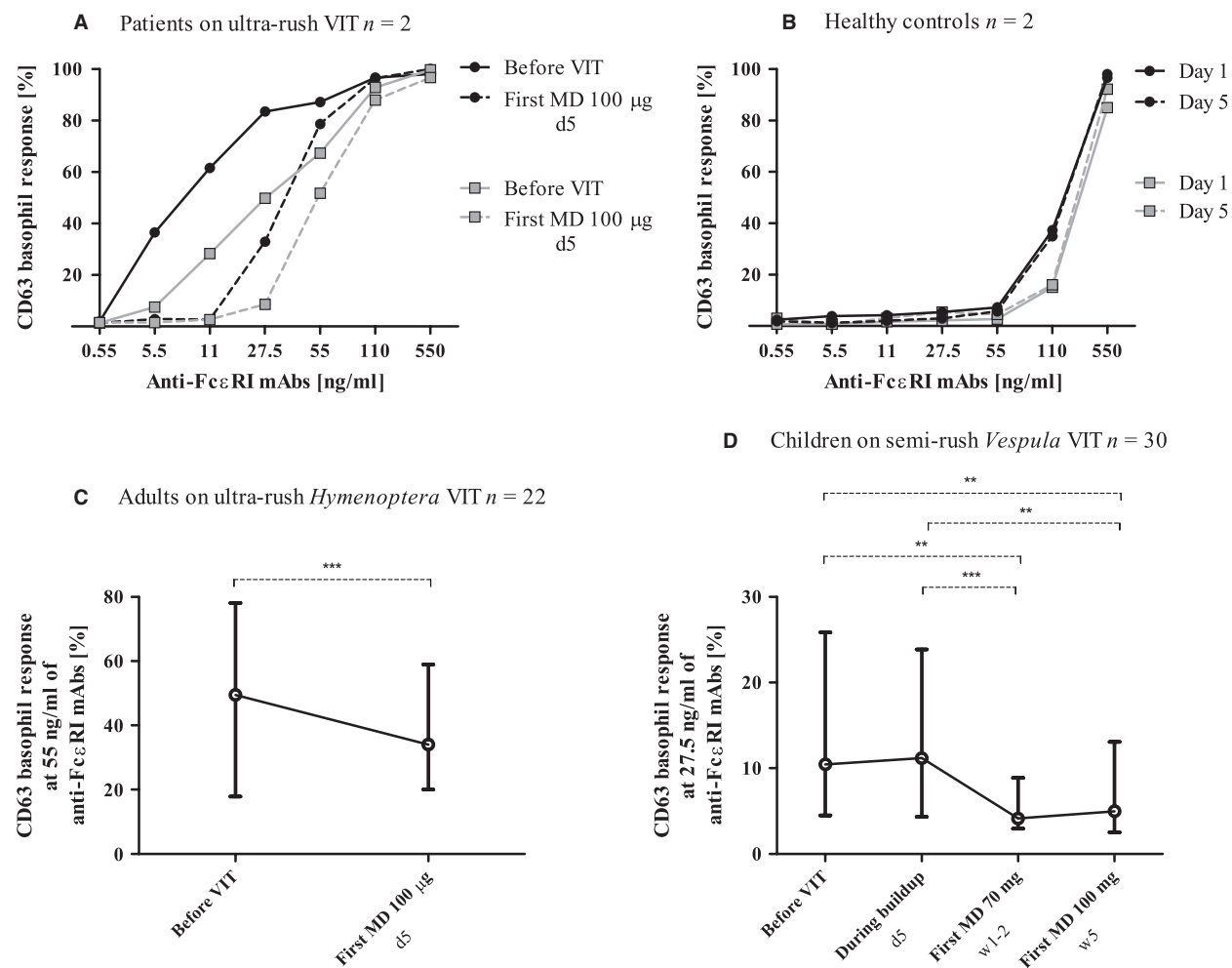


Figure 2 (A–D) CD63 basophil response in A two patients before ultra-rush VIT and the first MD of 100 μg and in B two healthy controls on days 1 and 5. CD63 basophil response at 55 ng/ml of anti-FcεRI mAbs in C 22 adults before ultra-rush VIT and the first MD of 100 μg and at 27.5 ng/ml in D 30 children before semi-rush VIT,

during buildup, before the first MD of 70 and of 100 μg . Data are presented as dose-response curves or median values with IQR after stimulation with serial dilutions of anti-FcεRI mAbs. ** $P < 0.01$ *** $P < 0.001$

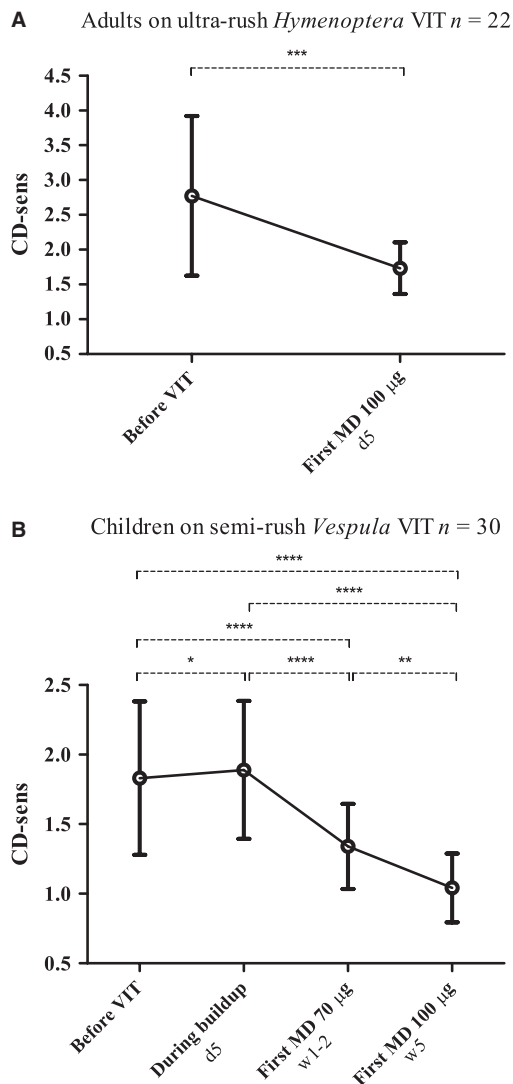


Figure 3 (A, B) Basophil threshold sensitivity (CD-sens) to anti-FcεRI stimulation in A 22 adults before ultra-rush VIT and the first MD of 100 µg and in B 30 children before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. Data are presented as mean values with 95% CI of mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

Stimulation with submaximal concentration of anti-FcεRI

The CD63 basophil response following stimulation with submaximal concentrations from 110 to 5.5 ng/ml decreased significantly in both groups of patients. In adults, the decreases before the first MD of 100 µg were the most significant when tested at submaximal concentrations of 55 ng/ml (median 34.1%; IQR 20.1–59.0), 27.5 ng/ml (3.9%; 2.3–7.3), and 11 ng/ml (2.6%; 2.1–3.8), when compared to before ultra-rush VIT (for 55 ng/ml: 49.5%, 17.9–78.1, $P < 0.001$, Fig. 2C; for 27.5 ng/ml: 8.1%, 4.1–27.7, $P < 0.001$; and for 11 ng/ml: 4.7%, 2.9–24.7, $P < 0.001$).

In 30 children, the decreases before the first MD of 70 and 100 µg were the most significant at submaximal concentra-

tions of 55 ng/ml (34.1%; 10.0–67.6; $P < 0.05$ and 26.9%; 8.7–46.9; $P < 0.001$, respectively) and 27.5 ng/ml (4.1%; 3.0–8.9; $P < 0.01$ and 5.0%; 2.5–13.1; $P < 0.01$, respectively) when compared to before semi-rush VIT (for 55 ng/ml: 39.4%; 13.0–68.6 and for 27.5 ng/ml: 10.5%; 4.5–25.9), whereas they slightly increased during buildup (for 55 ng/ml: 49.0%; 13.9–71.8; and for 27.5 ng/ml: 11.2%; 4.3–23.9; Fig. 2D).

The CD63 basophil response at maximal concentration of 550 ng/ml was comparable between all time points for both study groups (adults: median 96.9% and 96.7%, children: median between 90.4% and 91.6%). Similar response was found for minimal concentration of 0.55 ng/ml (adults: median 1.9% and 1.5%, children: median between 3.4% and 2.2%).

The group of healthy controls had similar responses on days 1 and 5 at each of the concentration tested (the differences were less than 5%; $P > 0.05$).

Basophil threshold sensitivity to anti-FcεRI stimulation (CD-sens)

Second, CD-sens, a measure of basophil threshold sensitivity to anti-FcεRI mAb stimulation, was estimated for both study populations and healthy controls. CD-sens in adults decreased significantly before the first MD of 100 µg (mean 1.7; 95% CI 1.4–2.1) in comparison with before ultra-rush VIT (2.8; 1.6–3.9; $P < 0.001$, Fig. 3A). Similar decrease was found in children before the first MD of 70 and 100 µg (1.3 and 1.0; 1.0–1.6 and 0.8–1.3; $P < 0.0001$, respectively) vs. before semi-rush VIT (1.8; 1.3–2.4), while CD-sens slightly increased during buildup (1.9; 1.4–2.4; $P < 0.05$, Fig. 3B). The decreases in CD-sens before the first MD were evident in all included subjects (100%).

In healthy controls, no discrepancy in CD-sens between the time points was noticeable (day 5: 0.7; 0.2–1.1 vs. day 1: 0.7; 0.2–1.2).

FcεRI gene expression profiles

FcεRI gene expression was analyzed on whole-blood samples of 60 adults and 18 children on ultra-rush VIT, in 30 children on semi-rush VIT, and in 5 healthy controls.

We found a significantly reduced expression of FcεRI gene before the first MD of 100 µg of ultra-rush VIT vs. before treatment, both in adults and in children ($P < 0.001$, Fig. 4A–B). The decrease was evident in 63% of adults and in 100% of children. However, in semi-rush VIT, FcεRI gene expression levels, when compared to before VIT, were significantly reduced only during buildup ($P < 0.01$, in 67% of subjects), and no differences were found before the first MD of 70 or 100 µg (to 34% of subjects; Fig. 4C). In control group, FcεRI expression remained at the same level on day 5 as it was on day 1 ($P > 0.05$).

To assess whether the decline in FcεRI gene expression is cellular related, human basophils were isolated in six adults before ultra-rush VIT and before the first MD of 100 µg. There were a decrease in four patients and a slight increase in two (Fig. 4D).

FcεRI cell-surface expression

FcεRI cell-surface expression was analyzed in 30 children on semi-rush VIT. We found a significant decrease before the first MD of 70 μg (mean 120×10^3 mol per cell; 95% CI $103\text{--}137 \times 10^3$; $P < 0.05$, in 59% of subjects), but no changes during buildup (136×10^3 ; $115\text{--}156 \times 10^3$) and before the first MD of 100 μg (135×10^3 ; $119\text{--}150 \times 10^3$), when compared to before VIT (127×10^3 ; $111\text{--}144 \times 10^3$, Fig. 5).

Absolute basophil cell count

Absolute basophil count was performed in 19 adults on ultra-rush VIT and in 30 children on semi-rush VIT. The blood basophil numbers before the first MD of 100 μg in ultra-rush protocol (12 basophils per μl; IQR 7–18) were highly comparable to prior to treatment (14 basophils per μl; 10–20, Fig. 6A). Absolute basophil count in semi-rush VIT; at the level of the first MD of 70 μg and 100 μg (19 and 15 basophils per μl; 11–30 and 10–25; respectively) was also comparable to before treatment (21 basophils per μl; 12–32); however, it decreased during buildup phase (11 basophils per μl; 8–18; $P < 0.001$, Fig. 6B).

Discussion

Venom immunotherapy unambiguously remains the treatment of choice for the prevention of severe systemic allergic reactions induced by *Hymenoptera* stings (22–24). It has been proven to be effective as soon as the MD is achieved (1,2), but the mechanisms responsible for the early protection have not yet been explained in detail (25). This study showed that short-term VIT induced a marked desensitization of FcεRI-mediated basophil activation, which seems to be associated with down-regulation of FcεRI expression.

Basophil desensitization was evident before the first MD, within 5 days of ultra-rush or a few weeks of semi-rush VIT, but not during buildup phase. These changes are comparable with previous observations that demonstrated decreased IgE receptor-induced histamine and sulfidoleukotriene release. Pinerkes et al. (11) proposed that this effect is partially due to the induction of IL-10 and IFN-γ production of T cells, and Novak et al. (12) suggested that desensitization could be rapidly induced by means of up-regulation of histamine receptor 2. Our results show that clinically induced basophil desensitization could also be associated with the reduction in FcεRI expression, and this observation may provide an additional explanation for the early effect of VIT. This correlates with a

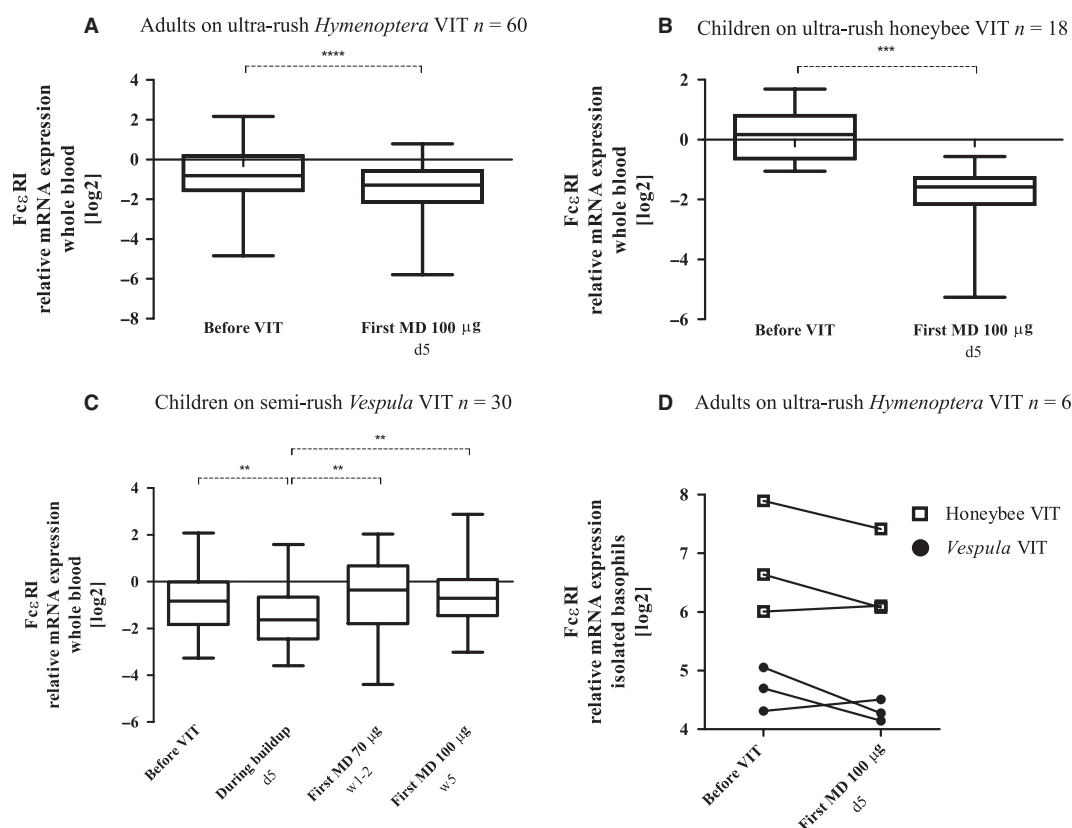


Figure 4 (A–D) FcεRI relative mRNA expression profiles of whole-blood samples in A 60 adults and B 18 children before ultra-rush VIT and the first MD of 100 μg and in C 30 children before semi-rush VIT, during buildup, before the first MD of 70 μg and of 100 μg and of isolated basophils in D 6 adults

before ultra-rush VIT and the first MD of 100 μg. In A–C, the box plot indicates the median (horizontal line), interquartile range (box), and minimal and maximal values (whiskers), and in D, data are presented as before–after plots. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

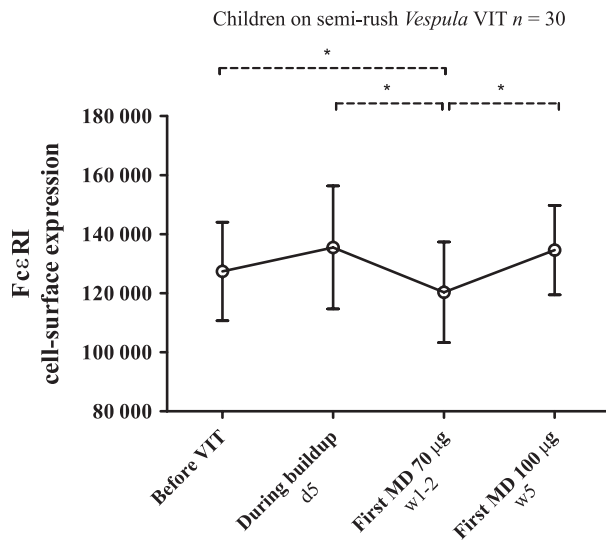


Figure 5 FcεRI cell-surface expression in 30 children before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. Data are presented as mean values with 95% CI of mean. * $P < 0.05$.

recent study of MacGlashan, which demonstrated that under conditions of *in vitro* subthreshold stimulation, basophils become desensitized due to the loss of FcεRI expression (17). In this study, desensitization leads to the loss of Syk as well; however, priming with IL-3 completely blunted the loss of Syk, but showed no effect on FcεRI.

While the FcεRI-mediated basophil desensitization was similar, both in ultra-rush and in semi-rush VIT protocol, there was a discrepancy concerning the FcεRI expression. Namely, the decrease in whole-blood FcεRI gene expression was significant only in ultra-rush protocols. In a limited number of patients, this was also confirmed at the level of isolated basophils. In semi-rush protocol, whole-blood FcεRI gene expression did not show any significant changes, although we demonstrated a significant decrease in FcεRI cell-surface expression. The observed discrepancy in gene expression might be influenced by the number of basophils. However, the blood basophil numbers at the MD level in

ultra-rush and semi-rush protocol were comparable to the state before starting VIT and were decreased only during buildup phase. Similar decreases in blood basophil count during buildup phase (12, 26) and returning to baseline values at the time of the first MD, after 1 week of immunotherapy, were also demonstrated previously (26). Early basophil desensitization seems to be a dynamic process that in different points might include different changes in the sensitivity of FcεRI signaling elements (14, 17, 27). Nevertheless, the differences between ultra- and semi-rush protocol might be also influenced by the cellular kinetic of basophils, which is relatively rapid with the turnover of several days (28). Interestingly, a similar kinetics as in ultra-rush VIT protocol was also demonstrated in anti-IgE treatment model, in which a significant FcεRI expression and basophil phenotype alternations were evident within 7 days of the initial treatment (15,16).

Although we did not assess the rate of clinical protection by sting challenge, all patients completed the buildup phase and reached the MD of 100 µg of venom. In fact, none of the previous (3,10–13) or the current study of early VIT-related markers objectively assessed the rate of early clinical protection as shown previously (1,2). We recently demonstrated in our 8-year follow-up study that after VIT a significant and approximately fourfold decrease in allergen-specific basophil response was demonstrated in all tolerant subjects, which suggests that basophil changes could be crucial for successful induction of tolerance after completing a successful course of VIT (29). Similar allergen-specific basophil changes were also observed after 2–4 years of children honeybee VIT (30). Unfortunately, these studies involved only anti-FcεRI response to maximum stimulation (at 550 ng/ml), and thus, we were not able to assess whether FcεRI-mediated subthreshold basophil desensitization also appears in later course of VIT. In contrast, in current study, we did not assess the allergen specificity of FcεRI-mediated desensitization, that is, if beyond VIT allergen, these changes are also relevant for possible non-VIT allergens. Obviously, both those aspects should be clarified in further studies.

In summary, FcεRI-mediated basophil desensitization may be an important factor for inducing the early protective state

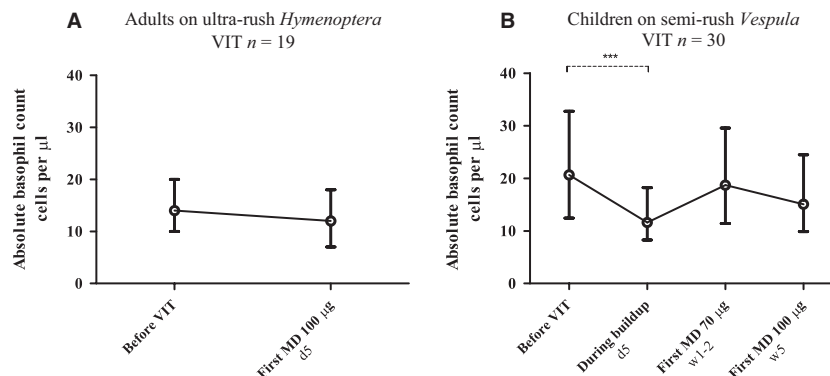


Figure 6 (A, B) Absolute basophil count (CD123 + /HLA-DR - cells) in A 19 adults before ultra-rush VIT and the first MD of 100 µg and in B 30 children before semi-rush VIT, during buildup, before the

first MD of 70 µg and of 100 µg. Data are presented as median values with IQR. *** $P < 0.001$

that must be achieved before the application of the first maintenance dose of VIT. Furthermore, a detailed understanding of these mechanisms would allow the development of novel interventions for promoting or monitor the silencing of basophil FcεRI pathway.

Authors' Contributions

P.K., N.Č. involved in the conception and design of the manuscript; N.Č., T.V., M.R., M.Š., R.E., M.K., S.E.K.Ž., and

T.A. involved in the analysis and interpretation of the data; P.K. and N.Č. involved in drafting the manuscript for important intellectual content.

Conflicts of interests

All authors declare that there is no conflict of interest.

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Supplemental data Fig. 1: Sequence analyses of IgE-derived VH after cloning. The N-terminal residues of the CH1 verify the IgE background of the obtained sequences.

[illegible]

Supplemental data Fig. 2: Sequence analyses of Bet v 1-specific scFv

Numbering and determination of the CDR was performed according to Kabat (21).

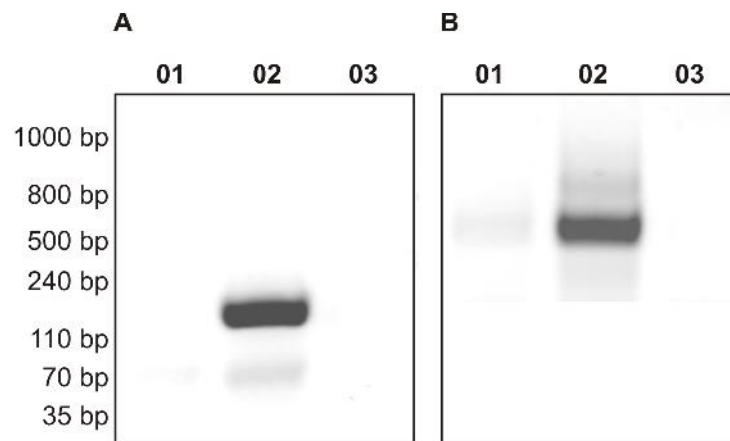
Alignments to V-Base (MRC, Cambridge) revealed usage of a VH3 and a VL1 segment.

```
VH
 1      10      20      30      40      50      60      70      80      90      100     110
B1 EVQLVESGGGLVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDN SKNMLHLQMNSLRAEDTAVYYCAKFGTQSR RFD PWGQGTPVTV
B2 EVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNS.LREDTAVYYCAKFGTQSRKFDFW GQGTPVTV
      CDRH1      CDRH2      CDRH3

linker
SSGGGSGGGGSGGSAL

VL
 1      10      20      30      40      50      60      70      80      90      100
B1 QSVLTQPPS.ASGTPGQRVTISCSGSSN.IGSNYVYVYQQLPGTAPKLLIYRNNORPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLVL.DVFGGGTKLTVLG
B2 QSVLTQPPS.ASGTPGQRVTISCSGSSN.IGSNYVYVYQQLPGTAPKLLIYRNNORPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLGTLFLVFGGGTKLTVLG
      CDRL1      CDRL2      CDRL3
```

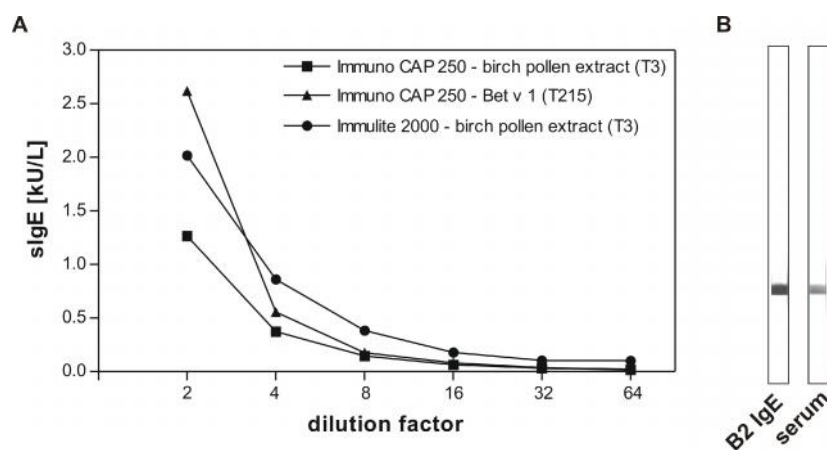
Supplemental data Fig. 3: Amplification of the VH from cDNA of three individual donors was performed using oligonucleotides specific for the CH1 region of IgE at the 3' end and a framework 3 specific oligonucleotide (A) and a framework 1 specific oligonucleotide (B) at the 5' end.



Supplemental data Fig. 4: Diagnostic application of recombinant IgE

A: Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE to immobilized birch pollen extract (T3) or Bet v 1 (T215).

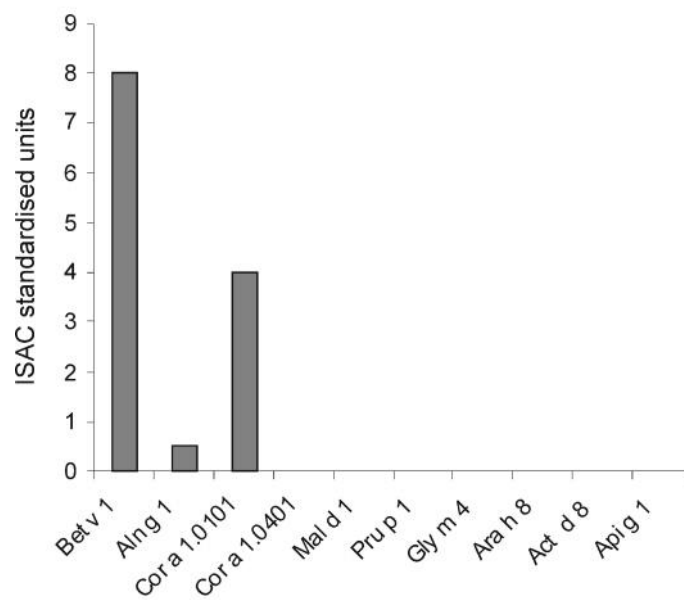
B: Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a pollen allergic donor to AlaBLOTs according to the manufacturer's recommendations. Detection was performed with anti human IgE conjugated to alkaline phosphatase.



Supplemental data Fig. 5: Alignment of the 37 amino acid residue C-termini of Bet v 1 isoforms (position 124-160) as available from the IUIS allergen database (excluding the highly variant isoform 01301).

Bet v 1.0101	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0102	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0103	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0201	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.0301	KGDQEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.0401	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0402	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0501	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0601	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0602	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0701	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0801	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0901	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1001	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1101	KGNHEMKAQIKASKEKAEALFRAVESYLLAHSDAYN
Bet v 1.1201	KGNHEMKAQIKASKEKAEALFRAVESYLLAHSDAYN
Bet v 1.1401	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1402	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1501	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1502	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1601	KGDHEINAEQIKIEKEKAEGLLKAVESYLLAHSDAYN
Bet v 1.1701	KGDHEVKAEQIKASKEMGETLLRAVERYLLAHSDAYN
Bet v 1.1801	KGDHEMKAHEMKAIKEKAEALLRAVESYLLAHSDAYN
Bet v 1.1901	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.2001	KGDHEMKAHEMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.2101	KGDHEINAEQIKIEKEKAVGLLKAVESYLLAHSDAYN
Bet v 1.2201	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2301	KGDHEVKEEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2401	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2501	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2601	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2701	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2801	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2901	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.3001	KGDHEVKAEQVKASKEMRETLLRAVESYLLAHSDAYN
	::*: *::* ** *::* * *****

Supplemental data Fig. 6: rIgE reactivity of PR10 proteins from pollen and food arrayed on the ISAC allergen chip.



Supplemental data Methods:

Reconversion into bivalent antibody formats and expression in mammalian cells

Recombinant immunoglobulins were produced using vector systems previously established in our lab (22). The antigen-binding regions were amplified using oligonucleotides containing restriction sites at the 5'- and 3'-termini of the VH (gatcatttaaattgtgtccagtgtgaggtgcagctgggtgg) and VL (gatcgcgatcgcaaccgaggacgggtcagct) by PCR, respectively. Subsequently, the DNA was introduced into the vectors for expression of the particular immunoglobulin isotypes.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich).

Assessment of immunoreactivity in ELISA and immunoblotting

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml diluted with 20 g/L MPBS) were applied to microtiter plates coated with particular antigens at 4 °C overnight and blocked with 40 g/L MPBS at RT for 1 h. Thereafter, the recombinant immunoglobulins (approx. 1 µg/ml diluted with 20 g/L MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and bound antibodies were detected using human Ig isotype specific antibodies conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm. The use of the soluble FcεRI-IgY Fc and FcγRI-IgY Fc in ELISA has been described elsewhere (1, 2). Briefly, allergen and immunoglobulins were applied as above. Bound antibodies then were detected using soluble FcεRI-IgY Fc and FcγRI-IgY Fc (approx. 1 µg/ml diluted with 20 g/L MPBS) and chicken IgG specific antibody antibody conjugate.

For detection of native allergen in immunoblotting procedures the recombinant IgE antibody was diluted using DMEM supplemented with 100 mL/L heat-inactivated fetal calf serum and applied at 1 µg/ml to AlaBLOTs (Siemens Healthcare Diagnostics), providing native allergens after separation by SDS-PAGE and transfer onto nitrocellulose membranes. Visualization of IgE was performed using anti-human IgE conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) as substrate. Reactivity with recombinant Bet v 1 isoforms and derived variants in immunoblot was performed after separation of proteins by SDS-PAGE. Recombinant IgE antibodies were applied and visualised as above.

rIgE reactivity of PR10 proteins arrayed on the ISAC allergen chip

The ISAC allergen chip data were obtained according to instructions of the manufacturer using rIgE containing cellular supernatant adjusted to a 200 ng/ml instead of human serum.

Generation of hybrid IgE repertoires

PBMC derived mRNA was prepared from the Trizol-solubilized samples after extraction with chloroform and precipitation with ice-cold isopropanol. cDNA was prepared by using Superscript III reverse transcriptase (Life Technologies) and constant region specific oligonucleotides. PCR was carried out using Pfx DNA Polymerase (Life Technologies) in combination with primers annealing to the 5'-end of the different IGHV subgroups and within the part of the IGHE gene encoding the constant domains of IgE (gtcaaggggaagacggatgggct). PCR-products were cloned and independent colonies subjected to sequencing.

Subsequently, VH-encoding genes were amplified from the PCR product using the 5' primers as above and the 3' primers annealing to the IGHJ genes according to VBASE and carrying restriction sites for SfiI and XhoI. The PCR-products then were used to replace the VH of the synthetic scFv-library Girffin 1 via SfiI and XhoI. Ligated DNA was electroporated

into *E. coli* TG1. Sequencing of randomly picked clones confirmed that the library carried members with a different genetic background.

Phage display selection of repertoire libraries

The selection of repertoires was essentially performed according to established protocols (3). Briefly, immunotubes coated with the particular allergen and blocked with 20 g/L MPBS were incubated with a total volume of 4 mL of 20 g/L MPBS containing 10^{12} to 10^{13} phages for 90 min under continuous rotation at room temperature. The immunotubes were then washed, 1 mL of 100 mmol/L triethylamine was added to elute the bound phages, and neutralization was performed by addition of 0.5 mL of 1 mol/L Tris-HCl, pH 7.4. After reinfection of *Escherichia coli* TG1 and overnight growth on agar plates, the phages were rescued by growth of scraped cells in liquid culture, infection with M13KO7 helper phage, and another period of overnight growth. Phages were then subjected to the next round of selection. Immunoreactivity of polyclonal or monoclonal phages was assessed in ELISA with anti-M13-horseradish peroxidase conjugate (GE Healthcare) and 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt for detection.

Construction of homology models

Homology models of Mal d 1 and grafted Mal d 1 were performed applying the SWISS-MODEL protein modeling server (4-6) and as a template the solved structures of Bet v 1 (PDB entry 1bv1) (7) and of the major cherry allergen Pru av 1 (PDB entry 1e09) (8), respectively. The quality of the models was confirmed by QMEAN4 score (Mal d 1: 0,62; grafted Mal d 1: 0,69) which is a combination of 4 statistical potential terms (9). Hydrophobicity and Coulombic surface coloring were performed using UCSF Chimera (10).

Recombinant production of Bet v 1 isoforms and derived variants

The PR10 protein Bet v 1a was amplified from cDNA using the sense oligonucleotide gatcgaattcgggtgtatttaattatgagactgagaccacc and the antisense oligonucleotide gatcaagctttcaatggtgatgggtgatgggtgtaggcacagagtgtg providing an Eco RI and a HindIII restriction site. The truncated fragments $\Delta 58$, $\Delta 91$, $\Delta 110$, and $\Delta 124$ were produced using the sense oligonucleotides gatcgaattcagctttcccagggtccctttcaagtacgtg, gatcgaattcccataggcgacacattggagaag, gatcgaattcgatggaggatccatcttgaagatcagcaac, gatcgaattcaaaggcgaccatgaggtgaaggcag, respectively. The Bet v 1 isoform was obtained from genomic DNA by use of the oligonucleotides gatcgaattcgggtgttttaattacgagagtgagac and gatcaagctttcaatggtgatgggtgatgggtgtaggcacagagtgtg.

Site directed mutagenesis of full length protein was performed using the antisense oligonucleotide atcggagtgtgccaagaggtagctccaac in combination with the Bet v 1a oligonucleotides mentioned above. On the basis of this template the corresponding fragment Bet v 1a $\Delta 124$ E 149G was generated as the $\Delta 124$ fragment.

The PR10 homologue from apple Mal d 1 was amplified from cDNA using the sense oligonucleotide gatcgaattcgggtgtctacacattgagaacgagtac and the antisense oligonucleotide gatccgtcgactcagtgatgggtgggtgatgggtgggtgtatgcgtcgggggtg providing an Eco RI and a HindIII restriction site. Epitope grafting into the C-terminus of Mal d 1 was performed using the antisense oligonucleotide ctttacttgccttaacctgctctgccttcacctcatggtcgcctttgggtg in combination with the Bet v 1a antisense and the Mal d 1 sense oligonucleotides mentioned above. On the basis of this template the corresponding mutant Mal L153/A154 was generated using the antisense oligonucleotide gatccgtcgactcagtgatgggtgggtgatgggtgggtgtatgcgtcgggggtgtgcaaaaggtagc. The amplicons were inserted into the prokaryotic expression vector pMal-2cX (NEB) and expressed according to the manufacturers' recommendations.

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