

A mutant of the major apple allergen, Mal d 1, demonstrating hypo-allergenicity in the target organ by double-blind placebo-controlled food challenge

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Summary

Background Allergen-specific immunotherapy for food allergy has been hindered by severe side-effects in the past. Well-characterized hypo-allergenic recombinant food allergens potentially offer a safe solution.

Objective To demonstrate hypo-allergenicity of a mutated major food allergen from apple, Mal d 1, *in vitro* and *in vivo*.

Methods A mutant of the major apple allergen, Mal d 1, was obtained by site-directed mutagenesis exchanging five amino acid residues. Fourteen patients with combined birch pollen-related apple allergy were included in the study. Hypo-allergenicity of the mutant rMal d 1 (rMal d 1mut) compared with rMal d 1 was assessed by *in vitro* methods, i.e. RAST (inhibition), immunoblotting and basophil histamine release (BHR) and *in vivo* by skin prick test and double-blind placebo-controlled food challenge (DBPCFC).

Results RAST analysis ($n = 14$) revealed that IgE reactivity to rMal d 1mut was twofold lower than that of the wild-type molecule (95% confidence interval (CI): 1.7–2.4). RAST inhibition ($n = 6$) showed a 7.8-fold decrease in IgE-binding potency (95% CI: 3.0–12.6). In contrast to this moderate decrease in IgE-binding potency, the biological activity of rMal d 1mut assessed by SPT and BHR decreased 10–200-fold. Hypo-allergenicity was confirmed by DBPCFC ($n = 2$) with both recombinant molecules.

Conclusion A moderate decrease in IgE-binding potency translates into a potent inhibition of biological activity. This is the first study that confirms by DBPCFC that a mutated recombinant major food allergen is clinically hypo-allergenic. This paves the way towards safer immunotherapy for the treatment of food-allergic patients.

Keywords apple, DBPCFC, hypo-allergenicity, Mal d1

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Introduction

Food allergies are an increasing problem in western countries [1]. Currently, up to 8% of children and 2% of the adult population are allergic to at least one food [2]. Treatment of food allergic patients is primarily based on avoidance diets. Such diets can have a considerable social impact and lead to nutritional deficiencies. For the treatment of inhalant allergies, specific immunotherapy was already described in 1900 by Curtis [3] and is now widely used as an effective treatment for pollinosis, animal, mite and insect venom allergy [4]. In contrast, specific immunotherapy is not commonly used to treat food allergies. A major problem of immunotherapy using food allergens is the risk of severe side-

effects, including anaphylactic shock. Although immunotherapy for food allergy was already described by Freeman in 1930 [5], it was not until 1992 that a controlled study with peanut-allergic patients was performed [6]. Using rush immunotherapy, clinical improvement of peanut allergy was found, measured by a decrease in symptoms during a double-blind placebo-controlled food challenge (DBPCFC) and a concomitant reduction in skin prick test (SPT) reactivity to peanut extract [6, 7]. Although this study indicated that immunotherapy can be effective in food allergy, the study was terminated prematurely, because a logistic error led to the administration of a too high dose of allergen, with fatal outcome. This tragedy stressed the need for safer approaches for allergen-specific immunotherapy. Recent developments in the field of allergen research offer new perspectives. Several studies have shown that it is possible to develop hypo-allergenic molecules of recombinant inhalant allergens. This was reported for Bet v 1 [8, 9], Lol p 5 [10], Lep d 2 [11] and Par j 1 [12]. Hypo-allergenicity was evaluated by *in vitro* and *in vivo* methods (SPT). Only in one study, hypo-allergenicity

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was confirmed in the target organ, i.e. by nasal challenges [9]. Many major food allergens have now been cloned and expressed as recombinant molecules. By site-directed mutagenesis, several major peanut allergens have been transformed into hypo-allergens [13–15]. Rabjohn et al. [14] showed by immunoblot analysis that IgE binding to modified Ara h 3 decreased by 35–85%, whereas it retained its capacity to induce T cell proliferation and activation. So far, mutant peanut allergens had not been assessed for hypo-allergenicity by *in vivo* methods like SPT and DBPCFC. One of the main reasons for this is the remaining risk of anaphylactic reactions. In this study, therefore, we chose to assess the general feasibility of the hypo-allergen approach by using a mutated major apple allergen Mal d 1, which is known to cause only mild symptoms [16]. In this way, we could safely evaluate biological activity by *in vivo* methods, avoiding the risk of serious side-effects. The deduced amino acid sequence of Mal d 1 shows 55% identity to Bet v 1 [17, 18]. Cross-inhibition experiments demonstrated that recombinant Mal d 1 shares IgE epitopes as well as T cell epitopes with the major birch pollen allergen, Bet v 1 [17, 19]. Recently, a new concept for the development of vaccines for specific immunotherapy (SIT) was established using the Bet v 1 molecule and introducing six point mutations at critical positions. The resulting molecule was still recognized by allergen-specific T lymphocytes but displayed significantly reduced IgE-binding capacity, as determined by *in vivo* and *in vitro* tests [20]. In the present study, we investigated whether the same amino acid residues/positions shown to be involved in the formation of IgE-binding epitopes of Bet v 1 are also crucial for IgE recognition and in particular the *in vivo* biological activity of Mal d 1.

Material and methods

Patients

Fourteen adult patients with a history of birch pollinosis and apple allergy were recruited via the outpatient clinic of the

University Medical Center Utrecht. Allergy was confirmed by a positive DBPCFC with apple, a positive skin test ($>7\text{ mm}^2$) and/or a positive RAST ($\geq 0.7\text{ IU/mL}$) for birch pollen and apple. All patients had seasonal rhinitis in the period of February–April, the tree pollen season in the Netherlands. Most patients were allergic to more than four different fruits and/or nuts in addition to apple (hazelnut, peach, pear, plum) (Table 1).

The study was reviewed and approved by the local ethical committee. Informed consent was obtained from all subjects before enrolment in the study.

Allergens

Natural Mal d 1 (nMal d 1) was purified by monoclonal antibody affinity purification [21]. The cDNA coding for recombinant (r)Mal d 1 (Mal d 1.0108, EMBL Genbank Database access no: AJ417551) was cloned into the pMW172 expression vector [22] as described previously [17, 23]. A hypo-allergenic mutant thereof, carrying five point mutations (T10P, I30V, T57N, T112C, I113V), designated rMal d 1mut, was designed and produced by analogy to hypo-allergenic Bet v 1mut [24]. Both rMal d 1 and rMal d 1mut were expressed in *Escherichia coli* strain BL21, insoluble inclusion bodies were solubilized in buffer containing imidazole and urea and subsequently dialysed to remove the urea. Subsequently, the extracts were subjected to further purification by DEAE chromatography by applying a $10 \times 100\text{ mm}$ column packed with DEAE-Sepharose Fast flow (Amersham, Biosciences, Little Chalfont, UK). Afterwards, the proteins were purified by separation over phenyl cellulose column ($10 \times 100\text{ mm}$; Biorad, Hercules, CA, USA). The proteins appeared homogeneous as single bands migrating at 17 kDa as determined by SDS-PAGE, and Coomassie staining and N-terminal sequencing revealed that the initiating methionine was cleaved off, resulting in the first five amino acid residues: GYVTF, respectively [25]. The integrity of the primary structure was further verified by MALDI-TOF mass spectrometry

Table 1. Clinical characteristics, SPT reactivity and RAST rMal d 1 and rMal d 1mut ($n=14$)

Patient number	Sex	Age (years)	Apple-related symptoms (CH)	SPT (mm^2)		RAST (IU/mL)		SPT (mm^2)		Ratio mutant $2\text{ }\mu\text{g/mL}$ (mm^2) SPT	RAST (IU/mL)		Ratio mutant RAST
				Apple	Birch	Birch	Apple	rMal d 1 $2\text{ }\mu\text{g/mL}$	rMal d 1mut $2\text{ }\mu\text{g/mL}$		rMal d 1	rMal d 1mut	
1	M	31	OAS RC	64	29	32.3	18.8	24	Neg	5	13.7	7.8	1.8
2	F	44	OAS RC	22	43	23.8	8.2	13	8	2	0.4	0.1	2.9
3	F	18	OAS RC CU	52	49	12.3	5.8	7	Neg	7	0.3	0.2	1.6
4	F	28	OAS	42	23	5.8	3.0	7*	Neg*	6	0.9	0.7	1.3
5	F	30	OAS RC CU	64	25	11.4	4.8	24	Neg	6	2.6	1.3	2.0
6	F	30	OAS	30	44	3.4	1.3	20	Neg	20	1.1	0.4	3.1
7	M	46	OAS	49	25	11.7	4.8	8	Neg	8	2.2	1.4	1.5
8	F	35	OAS RC	48	35	14.1	6.8	9*	Neg*	2	5.7	2.5	2.3
9	M	37	OAS	30	29	3.4	1.1	12	Neg	12	0.3	0.2	1.4
10	M	45	OAS RC CU	55	10	2.9	2.2	12	Neg	12	0.9	0.7	1.2
11	M	50	OAS	15	38	5.8	2.7	11	Neg	11	0.3	0.1	2.3
12	F	44	OAS RC	29	20	17.8	10.0	13	Neg	3	5.4	3.2	1.7
13	F	22	OAS RC CU	42	33	26.1	5.3	26	Neg	7	0.5	0.2	3.3
14	F	48	OAS RC	20	33	4.6	1.8	35	Neg	35	0.7	0.4	1.9

SPT, skin prick test; CH, case history; OAS, oral allergy syndrome; RC, Rhinoconjunctivitis; CU, contact urticaria; Neg, negative, rMal d 1, recombinant Mal d 1; rMal d 1mut, recombinant Mal d 1 mutant, M, male, F, female.

*20 $\mu\text{g/mL}$.

analysis. The molecular weight of reduced and alkylated Mal d 1wt and Mal d 1mut proteins was measured as 17556.65 (theoretical 17549.72) and 17576.87 (theoretical 17589.76), respectively [25]. Endotoxin levels were verified by the limulus amoebocyte lysate assay and found to be below the values allowed for human therapeutics for parenteral application [25].

Skin prick test with apple and recombinant allergens

Skin prick tests were performed in all 14 adult apple-allergic patients on the flexor surface of the forearm with fresh apple (Golden Delicious) using the prick-to-prick method [26]. They were also tested with purified rMal d 1 and the mutant thereof, rMal d 1mut. For optimal accuracy SPTs were performed in serial dilutions from 0.02, 0.2, 2, 20, 100 µg/mL of recombinant allergen. The SPT reactivity was measured after 15 min by copying the weal reaction with transparent adhesive tape onto a record sheet for later comparison. The skin weal areas were evaluated by computer scanning [27].

Basophil histamine release assay

White blood cells were isolated from the blood of a non-allergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere [28, 29]. Subsequently, cells were resensitized with the patients' serum. Histamine release was performed with purified rMal d 1 and rMal d 1mut (100 pg/mL–100 µg/mL). Liberated histamine was measured by the fluorometric method essentially as described by Siraganian [30]. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Centre under project number MEC 97/030.

Radio allergosorbent test and radio allergosorbent test inhibitions

For application in RAST, 100 µg purified nMal d 1 or rMal d 1mut was coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham-Pharmacia-Biotech, Uppsala, Sweden).

RAST was performed as described previously [31, 32]. Briefly, serum (50 µL) was incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 µL phosphate-buffered saline (PBS), 0.3% bovine serum albumin (BSA), 0.1% Tween-20 (PBS-AT). After washing away unbound serum components, radiolabelled sheep antibodies directed to human IgE (M1294, Sanquin, Amsterdam, the Netherlands) were added to a final volume of 500 µL in PBS-AT plus 4.5% bovine and 0.5% sheep serum (v/v) (Sanquin). After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units IgE per milliliters (IU/mL). Calculation was performed by means of a standard curve that was obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major house dust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2 [33]. A RAST value >0.3 IU/mL was regarded as positive.

For RAST inhibition, serum was pre-incubated with serial dilutions (200, 50, 12.5, 4.13 and 1.03 µg/mL) of inhibitor, rMal d 1 or rMal d 1mut (in 50 µL PBS-AT), prior to addition of Sepharose-coupled nMal d 1. For the uninhibited value, serum was pre-incubated with 50 µL PBS-AT. Sub-

sequent steps were identical to those described for the RAST. Results were expressed as percentage inhibition.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis /Immunoblotting

Proteins were separated by SDS-PAGE (NuPAGE[®] 4–12% Bis-Tris gel, protein: 10 µg/cm) according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA) and silver stained using a protein silver staining kit (Bexel Biotechnology, Union City, CA, USA).

Western blotting was performed by a semi-dry transfer onto nitrocellulose on a Novablot electrophoretic transfer apparatus, according to the protocol of the manufacturer (Invitrogen). After blocking with PBS/10 mM EDTA/0.3% BSA for a minimum of 10 min, blots were cut into strips prior to immunoprob- ing overnight with 150 µL human serum in 3 mL of PBS-AT. After washing away unbound serum with PBS/0.1% Tween-20, radiolabelled sheep antibodies against human IgE were used for detection of bound IgE. Blots were exposed to an X-ray film (Eastman Kodak Company, Rochester, NY, USA).

Double-blind placebo-controlled food challenge

The clinical reactivity of both the rMal d 1 and its mutant was investigated by DBPCFC in two patients using a procedure that has been described before [34]. In short, on the day of the challenge only a light breakfast was allowed, latest 2 h before the first test meal. Challenges were performed in a clinical research setting equipped for resuscitation and monitoring of vital signs. During challenges, the investigator or a nurse always accompanied the patient. The skin and oral cavity were inspected before the challenge for pre-existing lesions and during the challenge when symptoms like itching or a (feeling of) swelling occurred. Before and at regular intervals during the challenge, the blood pressure, heart rate and peak flow were measured for safety reasons and as parameters for detecting allergic reactions. An interval of 15 min was given between each test meal, or as long as was needed for (subjective) symptoms to disappear. Patients were kept for observation for at least 1 h after resolution of symptoms. The severity of the oral allergy symptoms, was scored using visual analogue scales (VASs) ranging from 0 to 100, where 0 equals no symptoms and 100 indicates severe symptoms.

The challenge drinks for provocation with recombinant allergens consisted of 500 µL of 10 mM Na acetate (pH 7) containing 0.01, 0.1 or 1 mg of each recombinant allergen. The three placebo doses consisted of 10 mM Na acetate. The active doses were randomly interspersed with three placebos.

A few weeks before the challenges with recombinant allergens, challenges with apple were performed to confirm clinical reactivity to apple. Each challenge meal with fresh apple was prepared within 5 min before administration and contained: 4, 10, 40 or 120 g of shredded apple (Golden Delicious), respectively. The meals were completed with a mixture of yogurt, orange juice, apple juice, applesauce, oatmeal flakes and dry rasped coconut. The placebo doses consisted of the same ingredients, except fresh apple. Apple juice and sauce do not contain any IgE-reactive Mal d 1 allergen because of processing steps and were added for optimal blinding of taste [35].

Statistics

For statistical evaluation of SPT and RAST with rMal d 1 and rMal d 1mut, the paired students-test was used. Differences associated with *P*-values of less than 0.05 were considered significant.

Results

Immunoglobulin E-binding potency

All 14 patients were tested by RAST with rMal d 1 wild type and its mutant, rMal d 1mut. The mean specific IgE to rMal d 1 was 2.5 IU/mL, ranging from 0.3 to 13.7 IU/mL. The mean specific IgE to rMal d 1mut was 1.4 IU/mL, ranging from 0.1 to 7.8 IU/mL. The mean ratio of IgE reactivity rMal d 1mut and rMal d 1 was 1.9 (95% confidence interval (CI): 1.5–2.3).

Six sera were used for RAST inhibition with rMal d 1 and rMal d 1mut. After parallel-line analysis of the inhibition curves obtained for each individual patient, a mean decrease in IgE-binding potency by a factor 7.8 (95% CI: 3.0–12.6) was calculated (Fig. 1).

All the sera were analysed by immunoblot with rMal d 1 and rMal d 1mut; four representative examples are shown in Fig. 2. By densitometric scanning, it was found that IgE reactivity to the rMal d 1mut was decreased by 25–45% compared with the binding to the rMal d 1 (individual values for the patients shown in Fig. 2 are indicated in the legend).

In vivo (skin prick test) and in vitro (basophil histamine release) biological activity

Recombinant Mal d 1 and rMal d 1mut were tested by titrated SPT in 14 patients. Parallel-line analysis of the

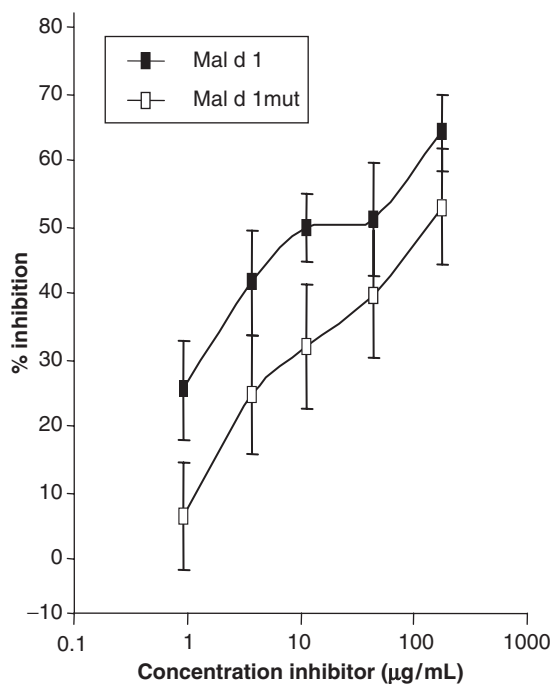


Fig. 1. The ability of rMal d 1 and rMal d 1mut to inhibit IgE reactivity to nMal d 1 sepharose was compared for six patients. This curve shows the mean values ($\pm$ SEM) obtained for each concentration used.

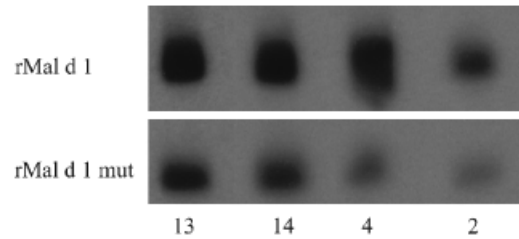


Fig. 2. Immunoblotting results with rMal d 1 and rMal d 1mut shown for four patients (patient numbers corresponding to Table 1). Individual decreases in IgE reactivity as measured by densitometric scanning are, in order of appearance, 34%, 37%, 38% and 43%, respectively.

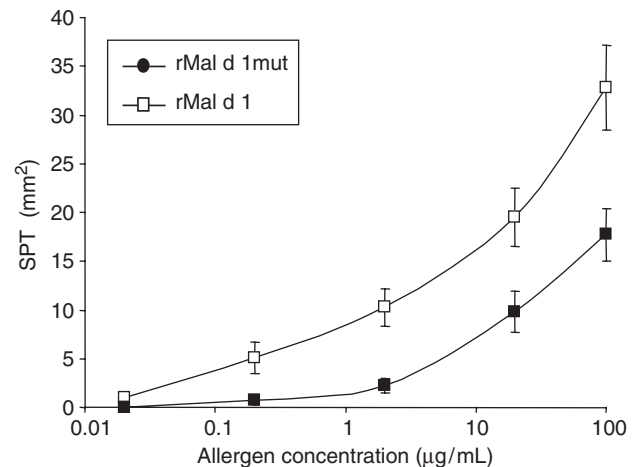


Fig. 3. Skin prick test (SPT) reactivity to rMal d 1 and its mutant. Results are shown as mean values $\pm$ SEM for *n* = 14 patients. **P* < 0.05 (paired *t*-test).

individual pairs of curves resulted in a mean decrease in the biological activity of the mutant molecule by a factor 28.4 (95% CI: 8.5–48.4), compared with the wild-type molecule. In concordance with this observation, the lowest concentration giving a positive SPT (> 7 mm²) was 2 µg/mL for rMal d 1 and 20 µg/mL for rMal d 1mut (Fig. 3).

The serum of three patients (3, 11 and 14) was used to sensitize stripped basophils. Upon incubation with either rMal d 1 or rMal d 1mut, the latter molecule demonstrated a 95-, 90- and 163-fold lower biological reactivity, respectively (determined by parallel-line analysis, Fig. 4). Compared with the decrease in IgE-binding potency (Table 3), the biological activity demonstrated a much larger decrease. To a lesser extent, this was also the case for the SPT reactivity.

Difference in the clinical reactivity of rMal d 1 and rMal d 1mut as determined by double-blind placebo-controlled food challenge

Two patients (nos. 1 and 2) underwent a DBPCFC with three 10-fold increasing doses of rMal d 1 and its mutant (Table 2). Both patients had a positive DBPCFC with fresh apple (Golden Del) (Fig. 5).

Patient 1 did not react to the first dose of rMal d 1 (0.01 mg), but reported typical oral allergy syndrome (OAS) symptoms within 5–10 min after ingestion of 0.1 mg rMal d 1. This included itching, a feeling of tightness in the oral cavity and tingling of the lips. Similar, but more severe symptoms

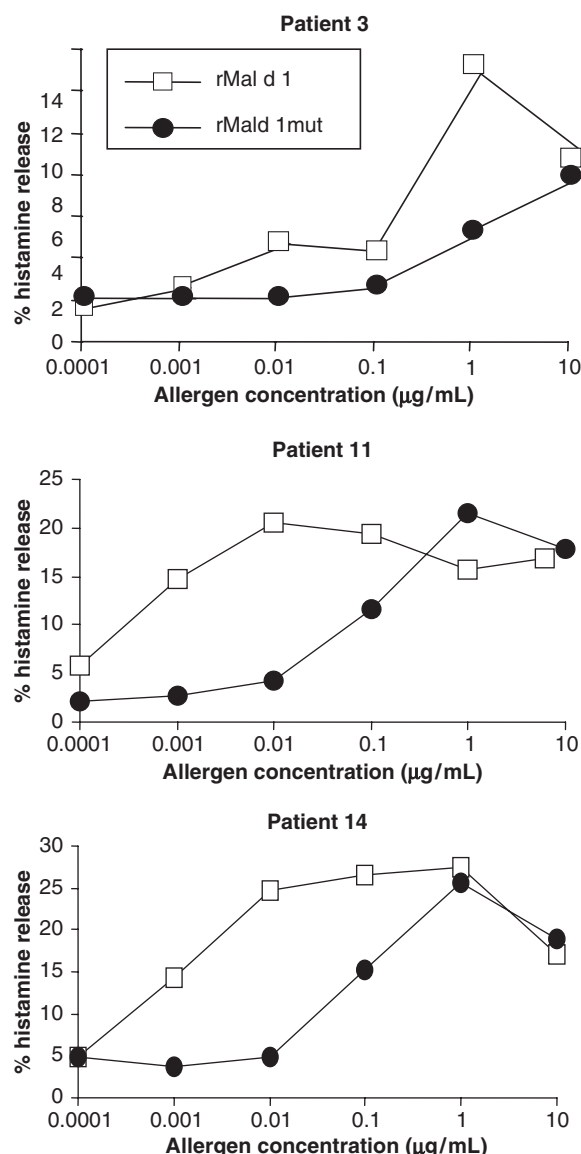


Fig. 4. Basophil histamine release. Comparison of biological activity of rMal d 1 and rMal d 1mut for three patients.

occurred at the higher dose of 1 mg. Reactivity to rMal d 1mut only occurred at the highest concentration tested (1 mg). No placebo reactions occurred.

Patient 2 reported typical oral allergy symptoms within 5 min after administration of the lowest dose of rMal d 1 given (0.01 mg). Similar, but more severe symptoms occurred after the higher dose of 0.1 mg rMal d 1. The highest dose of rMal d 1 (1 mg) induced hoarseness, coughing and dyspnoea (drop of 50% of peak-flow) and the patient was treated with inhaled β_2 -mimetic, oral antihistamines, and oral Prednisolone 40 mg, whereupon the symptoms gradually decreased. The medication was continued for another 2 days. Again, the mutant molecule induced symptoms only at 10-fold higher concentrations. These were limited to mild OAS symptoms. No hoarseness, coughing and dyspnoea were observed. No placebo reactions were reported. A summary of the *in vivo* and *in vitro* results given in Table 3.

Table 2. DBPCFC with rMal d 1 and its mutant in patients 1 and 2

Allergens	Dose (mg)	Reactions
Patient 1		
rMal d 1	0.01	0
Placebo		0
rMal d 1mut	0.01	0
rMal d 1	0.1	OAS 10
Placebo		0
rMal d 1mut	0.1	0
rMal d 1	1	OAS 60
Placebo		0
rMal d 1mut	1	OAS 10
Patient 2		
rMal d 1mut	0.01	0
rMal d 1	0.01	OAS 10
Placebo		0
rMal d 1	0.1	OAS 25
rMal d 1mut	0.1	OAS 10
Placebo		0
rMal d 1mut	1	OAS 40
Placebo		0
rMal d 1	1	OAS 60, Pf ↓ 50% hoarseness, coughing

rMal d 1, recombinant Mal d 1; rMal d 1mut, recombinant Mal d 1 mutant, DBPCFC, double-blind placebo-controlled food challenge.

Discussion

This is the first study demonstrating, by DBPCFC and SPT, that a recombinant food allergen can be made clinically hypo-allergenic. By mutating just five surface-exposed amino acids of the major apple allergen Mal d 1, the molecule lost at least 90% of its allergenicity. In the light of earlier reports that showed that hypo-allergenic peanut allergens can be hyper-allergenic for a minority of peanut-allergic patients [13–15], our results still need to be confirmed in a larger group of patients. Such variability would of course create a new safety issue, when immunotherapy with hypo-allergenic recombinant molecules is considered. Before the start of such a treatment, hypo-allergenicity should therefore always be confirmed on an individual basis. The stripped basophil test (or SPT) has proven to be a sensitive tool that could be used as a precautionary test for the assessment of biological activity prior to the start of treatment with hypo-allergens. The maximum levels of histamine release induced by Mal d 1 were relatively low. In our hands, Mal d 1 usually does not induce similar percentage release as e.g. Bet v 1 or LTP. Whether the maximum release indicates anything about the clinical relevance of an allergen is not (yet) known. The fact remains that histamine release starts at lower concentrations of the wild type than of the mutant Mal d 1. This is in agreement with the hypo-allergenicity observed in SPT and DBPCFC.

An interesting observation from this study is that a moderate decrease in IgE-binding potency (2–10 times) translates into a much stronger inhibition of biological activity (10–200 times). The most likely explanation for this apparent discrepancy is that mutagenesis has altered one or more IgE epitopes, resulting in decreased affinity of IgE for

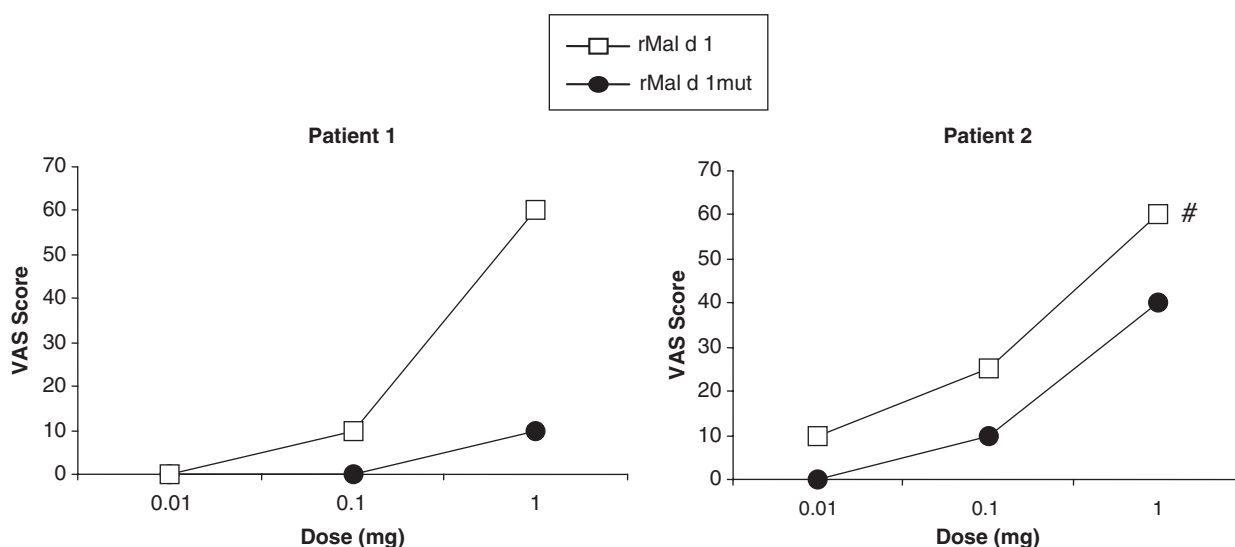


Fig. 5. Double-blind placebo-controlled food challenge with rMal d 1 and rMal d 1mut. #Hoarseness, coughing, dyspnoea with a 50% peak flow decrease.

Table 3. Comparison of *in vitro* IgE reactivity vs. biological activity

		Patient number		
		3	11	14
		Factor	Factor	Factor
SPT	rMal d 1 vs. rMal d 1mut (first reaction > HEIC 0.25)	100	100	100
BHR		95	163	90
RAST inhibition		2.8	7.9	4.6
RAST		2.0	1.7	1.8
Blotscan		1.4	1.5	1.8

SPT, skin prick test; BHR, basophil histamine release; rMal d 1, recombinant Mal d 1; rMal d 1mut, recombinant Mal d 1 mutant.

the allergen. This IgE will still bind to the allergen under saturating concentrations in a RAST, but will no longer bind strongly (i.e. long) enough to facilitate efficient cross-linking on effector cells. This implies that assessment of hypo-allergenicity by measuring IgE-binding potencies is not the best choice. Biological tests like BHR and SPT are essential here.

An unresolved question is how hypo-allergenic an allergen should be to be considered safe for application in immunotherapy. In other words, is a 10-fold decrease in allergenicity sufficient to prevent serious side-effects? This question is not easily answered, and also depends on the route of administration. On the other hand, there is great variability in the sensitivity of patients, which is, to a certain extent, reflected in the variation in their threshold at the minimum provoking dose. These have been studied for several foods like hazelnut, peanut, milk and egg [34, 36, 37]. Thresholds roughly ranged from 10 µg to 100 mg of these foods. In a food like peanut, the major allergens are storage proteins, which make up a large part of the total protein. This implies that threshold doses for individual peanut allergens most likely lie in the microgram to milligram range. Around the threshold dose severe reactions are not to be expected. Doses for sublingual administration will be 100–1000-fold higher.

Although tested in only two patients, an oral dose of 100 µg of the hypo-allergenic major allergen was well tolerated, and 1 mg caused only mild symptoms. In contrast a quantity of 1 mg of the wild-type allergen caused quite severe symptoms in one of these patients. In the light of recent studies where the total Mal d 1 levels in apple were estimated to be a maximum of 33.17 µg/g in the most allergenic cultivar [38], 1 mg would be the equivalent of 30 g apple. Whether these observations can be translated to peanut allergens is not clear. There is some evidence that threshold doses are lower for patients with severe peanut allergy than for those with mild allergy [36]. To be able to administer milligram quantities of major peanut allergen safely to patients with severe peanut allergy, most likely a larger reduction of allergenicity is required than has been achieved now for the major apple allergen.

Nevertheless, this study has provided proof for the feasibility of designing hypo-allergenic food allergens with thresholds higher than their wild-type versions. This will give new impetus to attempts to develop hypo-allergenic immunotherapeutics for food allergy.

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