Fruit allergy: from sensitization and symptoms to prevention and treatment

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ISBN 90-77595-78-3
Printed by: Optima BV Rotterdam

The research described in this thesis was financially supported by a grant from the EC: SAFE QLK1-CT-2000-01394.

Publication of this thesis was financially supported by:
J.E. Jurriaanse stichting, Kenniscentrum Voedselallergie TNO-UU, ALK-ABELLÓ, Pharma diagnostics, Fujisawa Holland BV, Allergy Consortium Wageningen (ACW) - The Netherlands, UCB Pharma, GlaxoWellcome BV, 3M Pharma, Galderma Nederland, HAL Allergenen, LEO Pharma BV, Bipharma, ACM OOMS Allergie BV.

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Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 14 september 2004 des middags te 16.15 uur.

door

Suzanne T.H.P. Bolhaar

Geboren op 12 maart 1974 te Venlo

Contents

1 General Introduction

Background

Adverse reactions to food can have many different underlying mechanisms ranging from toxic reactions and enzyme deficiencies to humoral and cellular immune reactions. In every-day language these adverse reactions are commonly referred to as food allergy. According to the 1995 position paper on food allergy of the European Academy of Allergy and Clinical Immunology, only immune-mediated hypersensitivities are to be designated as food allergy[1]. These include cellular reactions like celiac disease and IgE-mediated (or type I) allergic reactions. This thesis deals with IgE-mediated food allergy, more precisely with IgE-mediated fruit allergy. Food allergy can present itself as mild local reactions of the oral cavity, the so-called oral allergy syndrome (OAS)[2,3], but it can also (simultaneously) affect many other target organs like the skin (local or generalized urticaria, atopic eczema), the gastro-intestinal tract (cramps, diarrhoea, vomiting), nose and lungs (rhinitis and asthma) and the cardiovascular system (anaphylactic shock)[4,5]. Mild reactions and potentially life-threatening systemic reactions can be caused by the same food, even in the same patient. Of course, it is the risk of anaphylactic shock that has greatest impact on the quality of life of food allergic patients. Whether a mild local or severe systemic reaction is induced will depend on a complex interplay between IgE antibody specificity, affinity of the interaction between IgE and allergen, physico-chemical characteristics of the allergen and the actual health status of the patient. Treatment of food allergy is limited to avoidance and rescue medication. Effective avoidance can be difficult due to unintentional contamination of food products, incorrect or unclear labeling[6] and carelessness of food providers or consumers[7,8]. More effective strategies for the treatment of food allergy are therefore urgently needed. The importance of this is stressed by the fact that the frequency of food allergy is on the rise[9-11]. It is against this background that the European Commission decided to put food allergy on their research agenda. This thesis is part of a multicentre collaborative study, the SAFE project, funded by the EU Fifth Framework Program. The acronym SAFE stands for "Plant food allergies: field to table strategies for reducing their incidence in Europe". Apple allergy was chosen as a model system because apples are widely consumed in Europe and they frequently cause allergic reactions with a variable degree of severity[12]. The project aimed at addressing all aspects of food allergy from sensitization to clinical allergy, as well as at evaluating strategies for prevention and treatment.

Sensitization and cross-reactivity

It has been known for some decades that food allergy can be the result of primary sensitization to inhalant allergens. Allergies to plant foods are often linked to primary sensitization to pollen[2,13-17]. For several decades, apple allergy was almost exclusively seen as a phenomenon that occurs as a result of primary sensitization to birch pollen. In line with this, most early reports on apple allergy originate from countries where birch trees are abundant, like Scandinavia and Austria[18-26]. The basis of the co-occurrence of birch pollen and apple allergy is cross-reactive IgE. IgE antibodies against the major birch pollen allergen Bet v 1 cross-react with its homolog in apple, Mal d 1[26-30]. Bet v 1 and its homologs belong to a family of so-called pathogenesis-related (PR) proteins in plants, more specifically to

the PR10 family[16]. Apart from apple, IgE antibodies against Bet v 1 can cross-react to PR10 proteins in a broad spectrum of fruits from the *Rosaceae* family, in tree nuts like hazelnut and even in vegetables like celery and carrot. It was the clinical observation that birch pollen and fruit allergy go hand in hand that first led to the concept of cross-reactivity. *In vitro* confirmation of the existence of cross-reactive IgE antibodies was provided by RAST-inhibition (RI) assays[23]. In addition to establishing cross-reactivity, RI is an excellent tool to identify the primary sensitizer. IgE antibody binding to apple extract using serum samples from birch pollen allergic patients can be completely inhibited by birch pollen extract. The reverse is not the case, implying that the epitope spectrum of Bet v 1 is broader than that of Mal d 1.

A second cross-reactive allergen in birch pollen is Bet v 2 or profilin[31]. The apple homolog was designated Mal d 4 (AF129426/AJ507457). In contrast to Bet v 1, this allergen is also found in grass and weed pollen. Again pollen is the primary sensitizer resulting in cross-reactivity to an even broader spectrum of plant foods. Even human profilin has been reported as an (auto-) allergen in a limited number of pollen allergic patients[31]. Profilin is a highly conserved protein present in all eukaryotic cells, explaining its broad spectrum of cross-reactivity[26,32,33].

The clinical relevance of cross-reactivity to Mal d 1 is undisputed: up to 70 % of patients with Bet v 1-specific IgE have clinical apple allergy[2,15,25]. The clinical relevance of food profilin is most likely very limited in birch pollen allergic patients[34-36]. Profilin involvement in clinical fruit allergy has been reported mainly in grass pollen allergic patients from Spain[37,38]. The exact reason for this difference in clinical relevance is still not completely understood. The question to be answered is why IgE antibodies against cross-reactive pollen allergens demonstrate different degrees of biological activity. Most likely, anti-profilin IgE responses without clinical consequences have a more limited epitope spectrum and a lower overall affinity compared to those cross-reactive antibodies that do cause symptoms. Efficient mediator release can only be induced when multiple IgE epitopes are available and (at least some of) the IgE-allergen interactions are of high affinity[39].

Geographic differences in clinical presentation

In birch pollen allergic patients from Central and Northern Europe symptoms induced by fruit are almost exclusively mild and limited to the oral cavity[2,3]. In the early nineties it was first reported that patients with allergy to fruits such as apple and peach from Mediterranean countries frequently demonstrated more severe symptoms[40,41]. Furthermore, many of these patients were not allergic to pollen suggesting that sensitization in these cases is caused by ingesting fruit[40,42]. Spanish and Italian researchers identified and characterized the allergen that is responsible for fruit allergy in these patients, the so-called lipid transfer protein (LTP)[43,44]. Like Bet v 1 and its homologs, LTP is a member of a category of pathogenesis-related proteins, in this case of the PR14 family[16]. The allergen is preferentially expressed in the peel of fruits[16,45,46]. LTP in apple was designated Mal d 3[47]. It is now well-accepted that the high degree of resistance of LTP against proteolysis explains the severity of symptoms this allergen can cause[42,48-50]. In addition, the same stability is also thought to facilitate direct sensitization in the gastro-intestinal tract by this food allergen. Primary sensitization to pollen is not thought to play a role in LTP sensitization, although LTP has been identified

as an allergen in several allergenic pollen species, including mugwort[51-53] and *Parietaria*. From the latter pollen both major allergens, Par j 1 and Par j 2, are LTPs[54]. From most studies on LTP it becomes apparent that peach is clinically the most dominant food, suggesting that this fruit plays a role in LTP sensitization[40,41]. IgE antibodies against peach LTP (Pru p 3) have been shown to cross-react to LTP in related fruits like apricot, cherry and apple[55]. Furthermore, in selected patients a broader spectrum of cross-reactivity has been reported ranging from vegetables and tree nuts to cereals and seeds. Processing steps like cooking and pasteurisation do not significantly affect the allergenicity of LTP[42,48,49,56]. This explains why processed foods like juices, purees, and cooked vegetables and cereals (polenta) have been implicated in food allergy[48].

Another allergen with a similar stability profile was recently identified in fruits like apple and cherry. It is yet another PR protein, of the PR5 family, and it is structurally related to thaumatin[16]. It is therefore also referred to as thaumatin-like protein (TLP). TLP from apple was designated Mal d 2[57,58]. As is the case for LTP, TLP has been proposed to be a true food allergen, i.e. an allergen that can sensitize directly. Recently, TLP has been identified as an allergen in tree pollen[59,60]. Whether primary sensitization to pollen TLPs results in cross-reactivity to foods has not yet been established.

Diagnosis of fruit allergy

The gold standard of food allergy diagnosis is the double-blind placebo-controlled food challenge (DBPCFC)[61]. This situation is far from ideal because these challenges are laborious and not well suited to routine testing. In addition, some food allergens are rapidly degraded upon disruption of food tissue and last but not least, blinding to taste and/or texture can be difficult. Lability of food allergens is also the cause of the extremely poor sensitivity of commercial skin tests for fruits like apple and peach[62,63]. For this reason most skin tests for fruit are usually done by the prick-to-prick method[64]. Standardisation of this technique is of course difficult if not impossible. Unfortunately, the current generation of *in vitro* tests based on food extracts also fails to provide an optimal solution. Although the measurement of specific IgE against foods is on average technically sound and reliable, interpretation of the results is not always straight forward. A positive CAP or RAST for apple does not imply a positive diagnosis of clinical apple allergy[65]. Some IgE antibodies are of little or no clinical relevance[66]. This is frequently the case for IgE antibodies against plant glycans[67-70] also referred to as cross-reactive carbohydrate determinants (CCD) and for IgE against profilins[34]. Furthermore, the current tests for specific IgE do not distinguish between IgE against allergens that exclusively cause mild symptoms (e.g. Mal d 1) and that against allergens that potentially induce severe systemic reactions (e.g. LTP). The availability of well-characterized purified (natural or recombinant) food allergens has the potential to improve the clinical relevance of *in vitro* and *in vivo* IgE testing. Measurement of specific IgE against purified Mal d 1, Mal d 2, Mal d 3 and Mal d 4 could for example facilitate distinction of IgE antibodies with little or no clinical relevance from those linked to either mild or potentially severe clinical symptoms. An additional advantage of the use of purified allergens is that they are usually more stable than when the allergen is contained in fruit extracts, where phenol-oxidases, peroxidases and proteases can destroy their allergenicity[71,72]. Although

purified allergens are most likely a real improvement for the diagnosis of food allergy, tests that measure biological activity will also still be needed.

From avoidance to hypoallergenic foods

Apart from rescue medication, the only available therapy for food allergy is avoidance. Avoidance carries several disadvantages, ranging from possible dietary deficiencies to accidental ingestion due to inadequate labeling or contamination of foods[7]. The identification or development of hypoallergenic foods might provide an alternative to avoidance. It is frequently reported by apple allergic patients that they can tolerate some apple cultivars better than others[73,74]. These anecdotal reports on differences in allergenicity have recently been confirmed for a range of apple cultivars by in vitro experiments that showed differences in Mal d 1 activity[73,74]. These observations have initiated programs to unravel why these differences occur and how this knowledge can be used to breed new cultivars with low allergenicity by conventional crossing. So far differences in allergenicity have not yet been confirmed by DBPCFC. To really prove hypoallergenicity in vivo challenges are of course essential.

An alternative strategy to develop hypoallergenic apples is to inhibit the expression of Mal d 1 by RNA interference[75]. In that case the translation of messenger RNA is prevented by molecular biological techniques. These techniques have successfully been applied to plants. The first hypoallergenic plant produced in this way was rice[76]. So far, such genetically modified foods have not yet reached the marketplace and their hypoallergenicity has not been assessed by DBPCFC. Furthermore, acceptance of genetically modified foods has proven to be rather low in Europe, at least in part because modification was done primarily modified to increase production yields and revenues rather than improve food quality. It is important to investigate whether genetically modified foods with a clear benefit to consumer, like hypo-allergenicity, will enjoy a higher degree of acceptance.

Allergen-specific immunotherapy

Inhalant allergies, in particular those caused by pollen, can successfully be treated by allergen-specific immunotherapy[77]. Whether pollen immunotherapy also effectively reduces related cross-reactive food allergies is still a matter of debate, although most recent reports give an affirmative answer[78-81]. None of the studies was however based on double-blind trials and improvement of food allergy was not confirmed by DBPCFC. The treatment of true, non-pollen-related food allergy by allergen-specific immunotherapy is almost non-existent. The main reason for this is the high risk of serious side effects. The last published controlled trial to evaluate the potential of specific immunotherapy was performed in peanut allergic patients[82]. A high frequency of side effects requiring rescue medication characterised this trial and a tragic death due to human error resulted in a premature end to the trial. It is obvious that allergen-specific immunotherapy for food allergy only stands a chance if side effects can be controlled. State-of-the-art molecular biology now facilitates rapid and easy mutagenesis of major food allergens in order to reduce their allergenicity. In particular for peanut allergens like Ara h 1, 2 and 3 efforts have been made to obtain

hypoallergenic variants. *In vitro* experiments have indeed confirmed significantly reduced IgE-binding capacity[83-85]. It is most likely ethical considerations that have so far prevented *in vivo* confirmation of hypoallergenicity. Nevertheless, hypoallergenic approaches are probably the only way to introduce safe protocols for allergen-specific immunotherapy for food allergy.

SAFE and this thesis

The SAFE project funded by the EU aimed at addressing most of the issues mentioned above. Apple was used as the model system in these studies. This thesis is the result of clinical research performed within the framework of the SAFE project. *Chapter 2* describes the patient population studied by SAFE. Patients from four countries in Europe were included in SAFE, specifically from The Netherlands, Austria, Italy and Spain. Detailed IgE serology was performed using pollen extracts, food extracts and purified apple allergens. Results of these analyses were compared to clinical histories, skin tests and in some cases DBPCFC. The aims were to study the relation between sensitization to single allergens and clinical manifestations of food allergy, in order to establish the primary source of sensitization and resulting patterns of cross-reactivity and to draw conclusions regarding the possible improvement of diagnostic procedures for food allergy. In *chapters 3 and 4* two novel forms of cross-reactivity among birch pollen allergic patients are presented, i.e. to jackfruit and sharonfruit. In *chapter 5*, attention is given to the use of SPT and DBPCFC for the assessment of the allergenicity of different apple cultivars. *Chapter 6* describes the development of a hypoallergenic apple in which Mal d 1 is knocked out by RNA interference. This chapter is directly followed by a *chapter (7)* investigating the attitude of the general public and allergic patients towards hypoallergenic genetically modified foods. Finally, two chapters focus on strategies for allergen-specific immunotherapy. In *chapter 8* a birch pollen immunotherapy trial is described in which the effect on cross-reactive apple allergy is evaluated by SPT and DBPCFC. In *chapter 9*, a hypoallergenic mutant of the major apple allergen Mal d 1 is characterized by the same *in vivo* techniques. Mal d 3 certainly would have been a more important candidate to modify by mutagenesis, because this allergen is known to induce anaphylactic reactions in contrast to Mal d 1. Mal d 1 was, however chosen as a model allergen in this study to evaluate the general feasibility of the approach because oral challenges can safely be performed.

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2

Apple allergy across Europe: How allergen sensitization profiles determine clinical expression of plant food allergies

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Submitted

Abstract

Background: Allergy to a plant food can be the result of direct sensitization to that food, or of primary sensitization to pollen or to another food. In the latter two cases cross-reactivity of IgE is at the basis of the food allergy.
Objective: The aim of this study was to investigate which primary sensitizers are responsible for apple allergy across Europe, to establish which individual allergens are involved in sensitization and whether these differences have impact on clinical presentation.
Methods: Patients (n=389) were included in Utrecht (The Netherlands), Vienna (Austria), Milan (Italy) and Madrid (Spain) on the basis of a positive history for apple allergy and the presence of apple-specific IgE. A standardized questionnaire was filled out and skin prick test and radio allergosorbent test were performed for *Betula verrucosa*, *Phleum pratense*, *Olea europea*, *Artemisia vulgaris*, *Ambrosia elatior* and *Parietaria judaica* pollen as well as apple, peach, hazelnut, peanut, walnut, celery and carrot. In addition, all sera were tested for specific IgE antibodies against the apple allergens Mal d 1, Mal d 2 (thaumatin-like protein: TLP), Mal d 3 (lipid transfer protein: LTP) and Mal d 4 (profilin). Using SPSS, associations and correlations were analysed.
Results: Apple allergy in The Netherlands (NL), Austria (A) and Italy (I) is associated with birch pollinosis and sensitization to Bet v 1 and Mal d 1. In Spain (E), it is associated with allergy to peach and sensitization to LTP. Apple allergy starts at younger age in Spanish patients. The age of onset of apple allergy is around 7.5 years after the onset of pollen allergy in NL and A, and around 4.5 years in E and I. Only in Spain, the onset of allergy to peach coincides with that of allergy to pollen. Sensitization to Mal d 1 is a risk factor for development of local symptoms exclusively (OR: 3.3), whereas sensitization to LTP is a risk factor for systemic symptoms including generalized urticaria and anaphylactic shock (OR: 7.8).
Conclusions: Apple allergy in Spain is most likely a result of primary sensitization to peach. In NL, A and I it is a result of primary sensitization to birch pollen. Whether the low prevalence of LTP-related food allergy in these countries is caused by differences in consumption or in pollen exposure is still unclear.

Introduction

Allergy is sometimes referred to as the epidemic of the 21st century. Cross-sectional surveys carried out at different time points in the recent past have indeed demonstrated that the prevalence of respiratory allergies is rapidly growing[1,2]. In line with this increase, the prevalence of food allergies is expected to be on the rise as well. Epidemiological proof for increased prevalence of food allergy is so far scarce[3-6]. In large cross-sectional surveys like both European Community Respiratory Health Surveys (ECRHS I and II) and ISAAC, the prevalence of food allergy was not really addressed[4,7-9]. Similar large-scale studies focusing on food allergies are still missing, although some national surveys were performed[6,10,11]. Also prospective birth cohort studies that focus on the role of genes and environment in the development of allergy have so far mainly addressed respiratory allergies[12].

Reliable diagnosis is a prerequisite for epidemiology of food allergy. Based on public perception the prevalence of food allergy is overestimated considerably with Figures up to 30%[13]. The true prevalence is more likely to be between 1 and 10%, with higher Figures for young children than for older children and adults[13]. A prevalence of 1-2% among the latter and 6-8% among the former is generally accepted. Unfortunately these Figures are not very firm because techniques used to establish food allergy are not uniform and often have poor sensitivity and/or specificity. Questionnaire-based data can be coloured by the perception of food allergy by the general public, especially when they are not filled out under close supervision of clinical specialists. Skin prick tests (SPT) are commonly used by practitioners but many commercial food extracts have poor sensitivity[14-16]. This is in particular the case for fruits like apple and peach[17,18]. Prick-to-prick tests with fresh foods are more reliable but difficult to standardize and therefore not suitable for epidemiological surveys[19,20]. *In vitro* tests for specific IgE usually have excellent sensitivity but frequently lack specificity[21-23]. Detection of specific IgE by no means is a guarantee for clinical food allergy. Well-established examples of specific IgE antibodies without biological activity are those that were induced by primary sensitization to pollen and are cross-reactive to plant foods[24-26]. In particular cross-reactive IgE antibodies to plant N-glycans have been reported to lack clinical relevance[27].

In principle there are two pathways for the induction of food allergy, i.e. by way of primary sensitization to inhalant allergens or of primary sensitization to food allergens. It is now generally accepted that direct sensitization by food allergens is only possible when food allergens have high resistance to proteolysis in the gastro-intestinal tract[28-32]. The same property is thought to be decisive for the potential of food allergens to induce severe systemic reactions[28-31]. Two recently identified classes of allergens possess such extreme stability, the non-specific lipid transfer proteins (LTP)[28,33,34] and the thaumatin-like proteins (TLP)[35,36]. Both have a very compact structure stabilized by 4 and 8 conserved disulfide bridges, respectively[28,33-35]. Patients with IgE antibodies against LTP have indeed been reported to develop severe food anaphylaxis[28,33,34]. Although expected to be a similarly severe allergen, this has not yet really been firmly established for TLP.

So far, pollen-food cross-reactive IgE antibodies have mainly been implicated in mild local symptoms of the oral cavity or have been shown to even lack clinical relevance at al[21,22,24,26,37,38]. The main sources of cross-reactivity, the major birch pollen allergen Bet v 1[39] and the pollen profilins[40], have indeed been shown to be extremely sensitive to pepsin

digestion, explaining the restriction of symptoms to the oral cavity[28]. Similar to carbohydrate-specific IgE antibodies, those to profilin have been reported to lack biological activity in some patient populations[24,41,42]. Other reports have linked profilin to mild food allergy[21,43].

The availability of purified (natural or recombinant) food allergens of some of the most important plant foods[36,43-45] now provides the opportunity to drastically improve the predictive value of diagnostic tests. The measurement of IgE reactivity against individual allergens makes it possible to rank test results according the risk they bring along to induce severe food allergy. IgE antibodies against stable allergens like LTP will most likely demonstrate a much higher relative risk to be involved in the induction of severe systemic reactions than IgE antibodies against for example Bet v 1-homologues in plant foods. In contrast, IgE antibodies against profilins or plant N-glycans will carry only a small risk for inducing any clinical food allergy. Knowledge about IgE recognition profiles against purified allergens can also provide valuable information about the possible pathway of sensitization. It is essential for the development of preventive strategies for food allergy to know whether a food has caused direct sensitization or whether symptoms are a result of primary sensitization by pollen or by another food.

In this study we have chosen apple as a model to study the relation between sensitization profiles and the spectrum of clinical food allergies. Apple is one of the most commonly eaten fruits in Europe and the prevalence of apple allergy is significant[46,47]. In Northern and Central Europe, apple allergy has always been described as a mild disease[37,38,48]. More recent observations from Southern European countries indicate that apple can induce severe systemic reactions as well[49]. For apple, the most important allergens are available as purified natural or recombinant reagents, i.e. Mal d 1, Mal d 2 (TLP), Mal d 3 (LTP) and Mal d 4 (profilin). IgE reactivity to these allergens was measured by radioallergosorbent test (RAST) in serum of a total of 389 apple-allergic patients from four geographically and culturally distinct regions in Europe: The Netherlands (maritime/temperate climate), Austria (alpine/continental climate), Northern Italy (alpine/Mediterranean climate) and Central Spain (Mediterranean/continental climate). IgE profiles to the four apple allergens were correlated to a broad spectrum of pollen and food sensitizations evaluated by RAST and SPT and to clinical histories obtained by standardized questionnaire.

Materials and Methods

Patients' selection

Patients were selected in 4 countries across Europe: The Netherlands (NL), Austria (A), Italy (I) and Spain (E), from winter 2001 to summer 2003. Dutch patients were recruited at the Department of Dermatology/Allergology of University Medical Center Utrecht. Austrian patients were selected at the Allergy Clinic Reumannplatz in Vienna during 2001 and at the Medical University of Vienna in 2002 and 2003. Italian patients were selected at the Allergy Unit of Ospedale Caduti Bollatese, in Bollate (Milano). Spanish patients were selected at the Allergy Unit of the Fundación Hospital Alcorcón in Madrid. To enter the study patients had to refer immediate adverse reactions to apple ingestion together with a positive SPT to fresh *Golden Delicious* apple. The study was performed with the approval of the local ethics committees, and with consent of the patients (or their legal representatives).

Clinical evaluation

The same clinical evaluation was carried out for all the patients, and it comprised a medical history, SPTs and blood sampling. A complete and careful medical history was obtained for each patient to fully characterize the reported reactions to apple. Special attention was paid to the clinical description of the reaction, form of presentation of apple (fresh or processed), eliciting dose, time interval between intake and onset of symptoms, treatment needed for the resolution of the reaction, and age at onset of first apple-induced reaction. Symptoms at sites of close contact with apple during handling or consumption (oral symptoms, rhino-conjunctivitis, breathlessness, digestive complaints and contact urticaria) were designated as **local symptoms**. Generalized urticaria, anaphylaxis and anaphylactic shock were classified as **systemic symptoms**. Finally, the presence of associated food and pollen allergies was also investigated, and their full clinical descriptions and onsets were collected.

All the clinical information was collected following a standardized questionnaire specifically developed for this study by all the clinical participants, and it was translated into Dutch, German, Italian and Spanish for its administration in the clinical setting. The questionnaire was transferred to a database generated in Access software. An identification code was assigned to each patient to preserve confidentiality. The same code was used for the SPTs and serology data.

Skin prick test (SPT)

All individuals included in the study underwent SPTs with fresh *Golden Delicious* apple (peel and pulp separately) performed in duplicate following the prick-prick method[19,50]. If the SPT with apple was positive the patient was eligible for the study, and was subsequently skin prick tested with fresh peach, and commercial extracts of hazelnut, peanut, walnut, celery, and the pollens of *Betula verrucosa*, *Phleum pratense*, *Olea europea*, *Artemisia vulgaris*, *Ambrosia elatior*, and *Parietaria judaica*. Histamine hydrochloride (10 mg/ml) and saline served as positive and negative controls, respectively. SPTs were performed on the volar surface of the forearm using a standard 1 mm-tip lancet, following the recommendations of the EAACI[20]. The wheal areas were measured by planimetry in mm^2, and this value was included in the SAFE data base. A SPT was considered positive if the wheal area was 7 mm^2 (diameter 3 mm) greater than the negative control[20]. At each clinical centre, SPT were carried out by the same investigator for all patients. The food and pollen extracts, and the lancets were kindly provided by ALK-Abelló (Hørsholm, Denmark).

Allergen extracts

Pollen was extracted in water as described earlier. Food extracts were prepared essentially according to Björksten *et al.*[17] This protocol includes addition of polyvinylpolypyrrolidone (PVPP) and diethyldithiocarbamate (DIECA) to prevent loss in allergenicity due to poly-phenoloxidase, peroxidase and proteolytic activity.

Natural and recombinant allergens

Natural Bet v 1 and Mal d 1 was affinity-purified using monoclonal antibody 5H8 as described elsewhere[51]. Natural Mal d 3 was purified by ion-exchange and size-exclusion chromatography (manuscript submitted). Recombinant (r) apple thaumatin-like protein (TLP), rMal d 2, was produced in tobacco plants[36]. rMal d 4 were produced in *E. coli* (manuscript submitted).

Radioallergosorbent test

RAST was performed as described previously[52,53]. In short, allergen extracts and purified natural and recombinant allergens were coupled to CNBr-activated Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden). Food extracts (apple, hazelnut, peach, peanut, walnut, celery and carrot) were coupled at 40 mg per gram Sepharose, pollen extracts (*Betula verrucosa*, *Phleum pratense*, *Olea europea*, *Artemisia vulgaris*, *Ambrosia elatior*, *Platanus acerifolia*, *Chenopodium album* and *Parietaria judaica*) at 25 mg per gram and purified allergens (nBet v 1, nMal d 1, rMal d 2, nMal d 3 and rMal d 4) at 1 mg per gram. Serum samples (50 µl per test) were incubated overnight with 0.5 mg Sepharose (1.5 mg in case of food extracts) in a final volume of 300 µl PBS/0.3% BSA/0.1% Tween-20 (PBS-AT). After washing 5 times with PBS/0.1% Tween-20 (PBS-T), Sepharose was incubated overnight with ^{125}I-labeled sheep antibodies against human IgE (Sanquin, Amsterdam, The Netherlands) in a final volume of 800 µl PBS-AT, followed by 4 washing steps with PBS-T. Bound radioactivity was measured in a -counter. Results were expressed in international units (IU) IgE per ml, calculated from a standard curve of human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled rDer p 2[54]. For statistical analysis, a result > 0.3 IU/ml was regarded as positive unless stated differently.

For measurement of specific IgE against natural apple profilin (Mal d 4) Sepharose-coupled poly-L-proline was used to immobilize Mal d 4 from apple extract as described earlier (ref). The rest of the protocol was identical as described above. RAST analysis was centralized and performed at Sanquin Research, Amsterdam, by the same investigators.

Statistics

Statistical analysis was performed using SPSS and Epi Info software. Descriptive statistics included frequency of positive results (as percentage) with its 95% confidence interval (95%CI) for qualitative variables. For quantitative variables such as age, mean and standard error of the mean (SEM) were calculated, whereas for SPTs and RAST results median and 25 and 75 percentiles (P25, P75) were given. A chi-square test (with Yates correction if applicable) was used for comparisons of frequencies. Differences in quantitative variables between countries were compared by analysis of variance (age at study and age at onset) and median tests (SPTs and RAST results). To compare the ages at onset of pollen and food allergies within the same individual a Wilcoxon test for paired data was used. Clinical presentation was classified as local or systemic, and its association with the variables country, pollen allergy, and RASTs to Mal d 1, 2, 3 and 4 (categorized by their median values) was analysed by chi-square test, and the odds ratio with its 95%CI was calculated. Paired correlations were calculated by Pearson test ($r_{Pearson}$). Values were considered significant at $p<0.05$.

Results

Demographics

In this cross-sectional study 389 patients were included (Table 1), 152 males (39.07%, 95%CI: 34.19%-44.11%) and 237 females (60.93%, 95%CI: 55.88%-65.80%), with a mean age of 33.91 years (SEM 0.66). The difference in gender frequencies was significant ($p < 0.001$). With the exception of I ($p>0.05$), the gender difference reached significance for each country separately ($p<0.01$). In NL and A only patients with an age ≥ 18 years were included, whereas 4 Italian patients were adolescents below 18 years. The Spanish clinical group was the only one treating pediatric and adult allergic patients routinely, and all individuals with an age ≥ 6 years that fulfilled the inclusion criteria were included. Consequently, 25 out of 99 Spanish patients were below 18 years. The mean age of the 74 adult Spanish patients was nevertheless > 8 years lower than that observed in the other three countries ($p<0.001$). No differences in age were found between Dutch and Austrian patients ($p> 0.05$). The mean age of the adult Italian patients (n=93) was almost 4 years higher than of those from NL and A ($p< 0.05$).

Since the Austrian patients (n=94) were recruited at two clinical centres, it was investigated whether significant differences between both groups existed with respect to demographic composition and clinical history. The gender division was not significantly different, but the mean age of the patients selected at the Allergy Clinic Reumannplatz (n=35) was significantly higher than those selected at the Medical University (39.2 versus 31.4; $p=0.001$). No differences were found in the clinical presentation of apple allergy between these two subgroups ($p> 0.05$). SPTs and RAST results were compared between the 2 subgroups, and the only difference found was a higher SPT and RAST to apple ($p< 0.01$), and a higher RAST to birch pollen ($p=0.02$) in the patients recruited at the private clinic, with no significant differences for the remaining foods and pollen tested. The IgE responses to Mal d 1, 2, 3 and 4 tested individually did not differ among these subgroups ($p>0.05$). As the demographic background, the clinical presentation of apple allergy and the apple allergen profile were similar, all the patients were combined into one group for further analyses.

Table 1. Demographic data

	The Netherlands	Austria	Italy	Spain
N	99	94	97	99
Gender	29 M (29.30%) 70 F (70.70%)	36 M (38.30%) 58 F (61.70%)	47 M (48.45%) 50 F (51.55%)	40 M (40.40%) 59 F (59.60%)
Age: mean (SEM)	36.91 (1.02)	35.33 (1.17)	40.05 (1.45)	23.64 (0.93)

M: male; F: female; mean age given in years.

Clinical presentation

The clinical presentation of apple allergy in the four study countries is shown in Table 2. All patients reacted to fresh apple. Peeling or handling apples induced contact urticaria in some

patients, especially in NL (30%) and A (13%). The most frequent form of clinical presentation of apple allergy in all four countries was oropharyngeal symptoms classically known as the oral allergy syndrome (OAS), although its frequency was significantly lower among Italian and even more so among the Spanish patients. Digestive symptoms (gastric pain or burning as an immediate reaction after apple intake) were observed in less than 20% of patients and were generally preceded by OAS. Respiratory symptoms included rhinoconjunctivitis and asthma. Rhinoconjuntivitis appeared while biting and chewing fresh apple and was more frequently reported by the Dutch and Austrian patients. All other respiratory complaints reported were asthma-like symptoms and designated as "chest symptoms". They included a wide variety of manifestations like cough and wheezing dyspnea (in a minority of patients), a general feeling of chest discomfort, and most frequently mild and transitory breathlessness. The latter was recorded particularly in NL. Due to the heterogeneity of chest symptoms, comparisons among the four countries were not possible. Generalized urticaria and anaphylaxis were more frequently found among the Spanish patients. A single case of anaphylactic shock was reported for a Dutch patient.

Patients were classified according to their clinical presentation in two categories, i.e. those demonstrating exclusively local symptoms versus those having systemic symptoms with or without local symptoms (Table 2). The clinical picture of Austrian and Dutch patients did not differ ($p > 0.05$) with exclusively local reactions in more than 95% of the subjects. Also in Italy most patients did not demonstrate systemic reactions (>90%), but compared to NL and A the frequency of systemic reactions was higher. Apple allergy in Spanish patients was significantly severer than in any other country. The frequency of systemic reactions was 4-, 8- and 35-fold higher than in I, A and NL, respectively ($p<0.001$). There was a significant association (chi-square 65.02, $p <0.0001$) between systemic reactions induced by apple and living in Spain translating into an odds ratio (OR) of 11.65 (95% CI: 5.83- 23.28).

Table 2. Clinical presentation

		NL %	A %	I %	E %	Paired comparisons #
Apple induced symptoms	Contact urticaria	29.59	12.76	4.1	2.02	NL>A>I ≅ E
	OAS	100	96.80	89.69	79.79	NL ≅ A> I> E
	Digestive complaints	10.10	15.95	10.30	17.17	NL ≅ A ≅ I ≅ E
	Rhinoconjunctivitis	42.42	28.72	8.24	4.04	NL>A>I ≅ E
	Chest symptoms	20.20	5.31	3.09	7.07	Not applicable
	Urticaria	0	4.25	2.06	28.28	E> NL ≅ A ≅ I
	Anaphylaxis	1.01	0	0	13.13	E> NL ≅ A ≅ I
	Anaphylactic shock	1.01	0	0	0	NL ≅ A ≅ I ≅ E
Clinical pattern	Only local	98.98	95.74	91.75	64.64	NL ≅ A; NL>I; A ≅ I; E<NL,A,I
	Systemic	1.02	4.26	8.25	35.36	NL ≅ A; NL<I; A ≅ I; E>NL,A,I
Pollen allergy		96.96	95.74	95.87	87.87	NL ≅ A ≅ I >E

Significant differences ($p<0.05$) are shown by < or >, while non significant differences are indicated by ≅

Associations with pollen and food allergies

A history of pollen allergy was associated with apple allergy in more than 95% of patients from NL, A and I, but only in 88% of the Spanish patients ($p< 0.05$). There was a significant association (chi-square 35.86, $p <0.0001$) between systemic reactions induced by apple and the absence of a pollen allergy with an OR of 10.0 (95% CI: 4.11- 24.29). The pollen species inducing the respiratory allergy were different among countries (Table 3). Birch pollen allergy was present in 84% and 93% of the Dutch and Italian patients respectively, with a concomitant grass pollen allergy in half of them. In contrast, birch pollen allergy was never observed among the Spanish patients ($p<0.001$). In this group, grass pollen allergy was diagnosed in 93% of the patients (versus 47% in NL and 52% in I, $p<0.001$), with a concomitant olive pollen allergy in more than 70% of them. Olive pollen allergy was not seen in the NL and I ($p<0.001$). Austria was not included in this analysis because pollen allergies were not adequately documented for more than half of the patients.

Peach allergy was the food allergy most frequently associated with apple allergy in Spain (89.9%). Patients in NL, A and I reported significantly less adverse reactions to peach ($p<0.05$), i.e. in 70.7%. 69.9% and 66.0% of the cases, respectively. In NL and A, hazelnut was the food most frequently associated to apple allergy (73.7% and 73.3%, respectively), and the second one after peach in Italy (57.7%).

Table 3. Pollens involved in pollen allergy

	NL N= 96	I N= 93	E N= 87
Grass	1.04%	9.61%	20%
Birch	46.87%	42.30%	0%
Grass+birch	45.83%	42.30%	0%
Grass+olive	0%	0%	72.94%
Olive	0%	0%	2.35%
Other	6.26%	5.79%	4.71%

Age of onset of apple allergy

The age of onset of pollen and apple allergy was documented by the questionnaire (Table 4). Recall data were documented for all patients in the study except 16/99 from NL. For A, I, and E similar data were also collected for peach and hazelnut. In the analysis, patients under 18 years of age from I and E were excluded. The age at onset of apple allergy was different across Europe: Spanish patients were the youngest ($p< 0.001$), Italian patients were the oldest ($p<0.001$), and Dutch and Austrian patients started at a similar age ($p>0.05$). The age at onset of pollen allergies in NL, A and E was comparable (between 15 and 17 years), and significantly lower than in the Italian patients (29 years, $p<0.001$). For the 4 study countries the age at onset of pollen allergy was lower than the age of first apple reaction ($p< 0.05$). Peach and hazelnut allergy started at an earlier age than apple allergy in the Spanish patients ($p<0.001$), whereas no difference was found between peach and hazelnut onsets on the one hand and pollen onsets on the other. In contrast both peach and hazelnut allergies started later than pollen allergy in A and I ($p<0.001$).

Table 4. Age at onset of pollen and food allergies

	NL	A	I	E
Pollen	16.92 (1.16)	16.82 (1.43)	28.96 (1.54)	15.06 (0.99)
Apple	23.89 (1.13)	24.31 (1.35)	33.63 (1.42)	18.85 (1.14)
Peach	NA	19.52 (1.58)	30.73 (1.59)	14.49 (1.14)
Hazelnut	NA	19.76 (1.55)	33.80 (1.81)	15.57 (1.99)

Mean (SEM) is given in years; NA: Not available

Specific IgE assessed by SPT and RAST

All patients were skin prick tested for pollen and foods (Table 5). SPTs responses to histamine were significantly different among the 4 clinical centers (not shown), and therefore absolute values of SPTs were not used in the inter-country comparisons. Skin reactivity to apple and peach was assessed using fresh peel and pulp of both fruits (except for Austria where peach was not tested). Overall, SPT results for apple were higher in Italy, and for peach higher in Spain. In Spain and to a lesser extent in Italy, skin reactivity to peel was higher than to pulp. This was not observed in NL and A. As expected, no significant skin reactivity to birch was observed in Spain. In contrast, grass pollen (*Phleum pratense*) induced significantly higher skin reactions in Spain. Skin reactivity to hazelnut was again significantly lower in Spain than in the other three countries.

When analyzed by RAST, IgE titers to apple were also higher in Italy, although this difference only reached significance compared to Spain (Table 6). The higher SPT reactivity to peach observed in Spain was supported by the RAST results. This was also the case for the differences observed for SPTs to birch pollen, grass pollen and hazelnut. The median IgE reactivity to grass pollen was around ten times higher in Spain than in NL, A and I. Also IgE responses to *Artemisia, Parietaria, Chenopodium* and *Platanus* were higher in E.

Both by RAST and SPT it was demonstrated that in NL, A and I, IgE titers against birch pollen were higher than those to apple which in turn were higher than those observed to peach. In contrast, in Spain IgE reactivity to peach was significantly higher than to apple.

IgE responses against individual apple allergens

All sera were used for profiling IgE responses against four individual apple allergens (Figure 1). In NL, A and I, IgE reactivity to Mal d 1 was significantly higher than in Spain. In NL, A and I, 69/99, 79/94 and 66/97 patients had IgE titers ≥ 1.0 IU/ml. In these countries, IgE responses to the homologue of Mal d 1 in birch pollen, Bet v 1, were significantly higher with 87/97, 90/99 and 83/84 showing IgE responses ≥ 1.0 IU/ml, respectively. In Spain these frequencies were 16/99 for Mal d 1 and 5/99 for Bet v 1. IgE responses to Mal d 3 (LTP) were significantly higher in Spain than in the other three countries. In turn, Italy demonstrated higher IgE reactivity to this allergen than NL and A. Frequencies of IgE titers ≥ 1.0 IU/ml were 47/99 (E), 11/99 (I), 1/99 (NL) and 2/94 (A).

IgE reactivity to Mal d 2 (TLP) were significantly higher in E and I compared to NL and A, but they were generally low. Only 16/99 (E), 6/97 (I), 5/99 (NL) and 0/64 (A) demonstrated IgE titers ≥ 1.0 IU/ml. For Mal d 4 (profilin), IgE response were significantly higher in Spain

than in the other three countries. Frequencies of IgE titers ≥ 1.0 IU/ml were 41/99 (E), 29/97 (I), 13/99 (NL) and 8/75 (A).

In NL, A and I, IgE responses to apple showed strong positive correlations ($0.6 < r_{Pearson} < 0.9$; $p<0.001$) to those to birch, Bet v 1 and Mal d 1. Only in Spain, strong and significant correlations were found between SPT to apple peel and SPT to peach peel ($r_{Pearson}=0.77$; $p< 0.001$), RAST to apple and RAST to peach ($r_{Pearson}=0.85$; $p<0.001$), and RAST to Mald 3 and RAST to peach ($r_{Pearson}=0.60$; $p<0.001$).

Table 5. SPTs results

	Descriptives: median (P 25-P 75)				**Median test**
	NL	**A**	**I**	**E**	**Paired comparisons**
Apple peel	0.78 (0.55-1.25)	0.81 (0.45-1.31)	1.47 (1.01-2.02)	0.86 (0.47-1.73)	I > NL ≅ A ≅ E
Apple pulp	0.60 (0.43-1.06)	0.92 (0.46-1.25)	0.96 (0.66-1.40)	0.25 (0-0.53)	A ≅ I > NL > E
Peach peel	0.64 (0.26-0.95)		0.55 (0.19-1.03)	1.62 (0.67-2.55)	E > NL ≅ I
Peach pulp	0.58 (0.47-1.0)		0 (0-0.41)	0.59 (0.31-0.93)	NL ≅ E > I
Birch pollen	1.0 (0.61-1.33)	1.12 (0.81-1.51)	1.54 (0.97-2.24)	0 (0-0.33)	I > NL ≅ A > E
Phleum	0.56 (0.06-1.04)	0.87 (0-1.52)	0.66 (0-1.63)	1.20 (0.51-2.16)	E > NL, A, I; A > NL; A ≅ I; NL ≅ I
Olea	0.26 (0-0.55)	0.37 (0-0.93)	0.46 (0-1.12)	0.94 (0.31-1.50)	E > NL, A, I; I > NL; A ≅ I; A ≅ NL
Artemisia	0 (0-0)	0 (0-0.72)	0.18 (0-0.78)	0 (0-0.33)	I > NL, E; I ≅ A; E,A > NL; A ≅ E
Ambrosia	0 (0-0)	0 (0-0.40)	0.49 (0-1.20)	0 (0-0.23)	I > A ≅ E> NL
Parietaria	0 (0-0)	0 (0-0)	0 (0-0.33)	0 (0-0)	I > E > NL ≅ A
Hazelnut	0.50 (0.31-0.72)	0.70 (0.38-0.91)	0.71 (0.50-1.0)	0.20 (0-0.47)	E < NL, A, I; A ≅ I; A ≅ NL; I> NL
Peanut	0.20 (0-0.55)	0.31 (0-0.88)	0.67 (0.24-1.11)	0.38 (0-0.66)	I> NL, A, E; E > NL; E ≅ A; NL ≅ A
Walnut	0 (0-0)	0 (0-0)	0 (0-0.18)	0 (0-0.54)	I ≅ E > NL ≅ A
Celery	0.37 (0-0.74)	0.55 (0-1.0)	0.68 (0.34-1.13)	0 (0-0.36)	E < NL, A, I; A≅ I; A ≅ NL; I> NL

The variable used in SPTs was the ratio to the histamine response (allergen wheal area / histamine wheal area)

Individual allergens and clinical presentation

To assess whether IgE antibodies against individual apple allergens have different impact on the clinical presentation of apple allergy, IgE responses against the four allergens of all patients together were each divided in two groups: those with an IgE titer above the median, and those with an IgE titer under the median (Figure 2). The odds ratio for developing only local symptoms was 3.33 (95%CI: 1.73-6.41) for those patients with an IgE response to Mal d 1 > median. For patients with an IgE titer against Mal d 3 above the median, the odds ratio for developing a systemic reaction was 7.76 (95%CI: 3.87-15.56). No significant associations were found for Mal d 2 and Mal d 4.

Table 6. Serology results

	Descriptives: median (P25 - P75)				Median test
	NL	A	I	E	Paired comparisons
Apple	3.08 (1.51-5.74)	3.06 (1.57-6.87)	4.15 (2.12-7.79)	2.81 (1.25-5.48)	I> E; NL ≅A ≅ I; NL ≅ A ≅ E
nMal d 1	1.75 (0.79-3.27)	2.54 (1.27-5.53)	1.78 (0.54-4.08)	0.32 (0.19-0.71)	NL ≅ A ≅ I > E
rMal d 2	0.14 (0.11-0.24)	0.15 (0.09-0.22)	0.26 (0.15-0.38)	0.32 (0.18-0.83)	I ≅ E > NL ≅ A
nMal d 3	0.12 (0.09-0.20)	0.12 (0.06-0.16)	0.23 (0.14-0.37)	0.91 (0.32-3.38)	E > I > NL ≅ A
rMal d 4	0.28 (0.21-0.42)	0.23 (0.16-0.39)	0.31 (0.23-1.96)	0.69 (0.24-3.23)	E > NL ≅ A ≅ I
Birch pollen	10.19 (5.07-18.21)	15.36 (8.34-29.69)	11.07 (5.29-18.51)	1.08 (0.34-3.23)	A > NL ≅ I > E
nBet v 1	12.70 (6.03-27.73)	18.65 (9.53-42.72)	15.79 (6.80-26.67)	0.17 (0.11-0.30)	A> NL; A ≅ I; NL, A, I > E
Phleum	4.96 (0.73-21.47)	3.65 (0.56-40.21)	3.70 (0.36-19.79)	41.23 (8.79-100.48)	E > NL ≅ A ≅ I
Olea	1.0 (0.43-2.43)	2.92 (0.70-7.15)	1.15 (0.30-3.29)	4.62 (1.85-11.41)	E > A > NL ≅ I
Platanus	0.32 (0.22-0.75)	0.64 (0.25-1.04)	0.37 (0.19-1.71)	1.68 (0.54-4.18)	E > NL ≅ A ≅ I
Artemisia	0.48 (0.26-1.53)	1.09 (0.34-3.10)	1.05 (0.32-3.34)	1.67 (0.79-3.81)	E > A ≅ I > NL
Ambrosia	0.29 (0.22-0.45)	0.41 (0.21-1.17)	2.37 (0.29-9.64)	0.56 (0.29-1.29)	I > A ≅ E > NL
Parietaria	0.42 (0.33-1.06)	0.57 (0.24-1.13)	0.76 (0.39-3.89)	1.61 (0.66-3.91)	E > NL, A, I; I ≅ A; I > NL
Chenopodium	0.64 (0.37-1.87)	1.18 (0.69-3.72)	0.99 (0.50-2.09)	2.72 (1.04-4.77)	E > NL, A, I; A ≅ I; A > NL
CCD	0.36 (0.29-0.48)	0.25 (0.17-0.65)	0.39 (0.35-0.48)	0.47 (0.34-0.95)	E > NL, A, I; I > A; NL≅ I, A.
Peach	1.42 (0.80-3.40)	1.88 (0.97-3.25)	1.74 (0.90-5.43)	4.92 (2.45-13.78)	E > NL ≅ A ≅ I
Hazelnut	2.50 (1.13-4.99)	3.93 (2.18-6.97)	2.95 (1.72-6.13)	1.98 (0.67-5.33)	A > I > E ≅ NL
Peanut	1.58 (0.66-4.19)	2.80 (1.18-5.01)	1.94 (1.08-4.81)	2.73 (0.84-5.59)	A > NL; I ≅ E ≅ NL; A ≅ E ≅ I
Walnut	0.50 (0.31-1.49)	1.17 (0.62-1.90)	0.72 (0.43-2.41)	2.16 (0.61-5.05)	E > A > I ≅ NL
Carrot	0.52 (0.24-1.54)	1.31 (0.52-2.25)	0.92 (0.42-3.84)	1.64 (0.50-5.60)	E > NL, I; E ≅ A; A≅I; A > NL; NL≅ I
Celery	0.50 (0.29-1.38)	1.09 (0.60-1.94)	0.96 (0.51-2.82)	2.03 (0.66-5.45)	E > NL, A, I; A,I > NL; A ≅ I

Significant differences ($p<0.05$) are shown by < or >, while non-significant differences are indicated by ≅

Figure 1. Sensitization to apple allergens in The Netherlands (NL), Austria (A), Italy (I) and Spain(E)

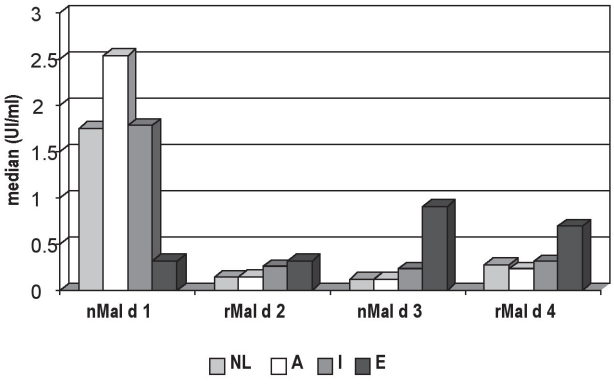
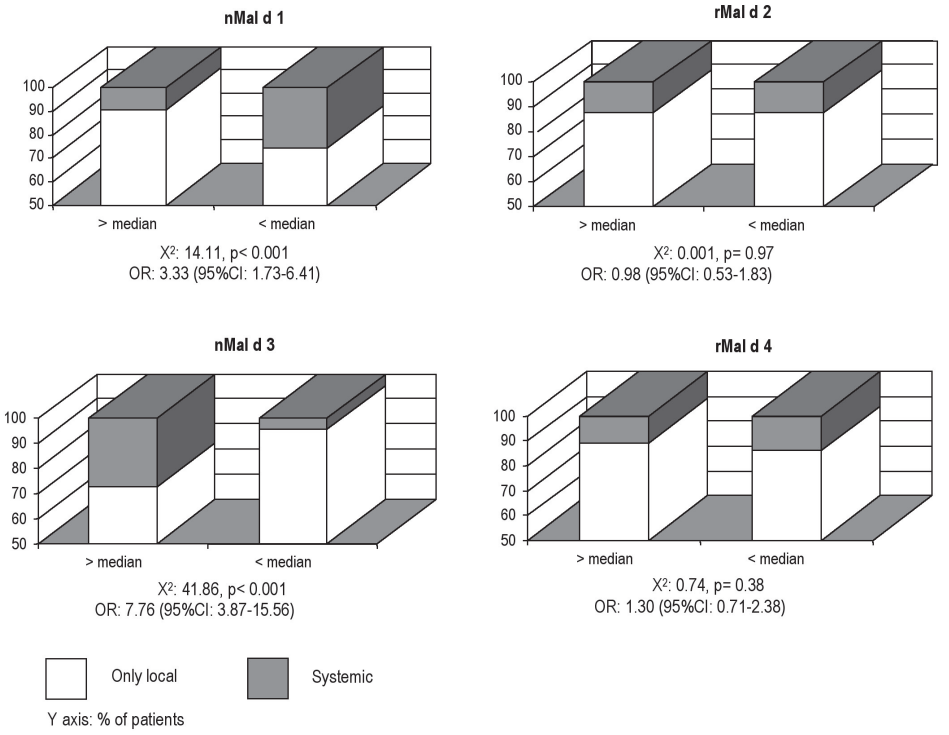


Figure 2. Sensitization to individual aplle allergens and clinical expression of apple allergy



Discussion

This study has analyzed the relation between sensitization to individual apple allergens and the resulting clinical presentation of adverse reactions to apple in a comprehensive multi-centre approach across Europe. In four clinical centres in Spain, The Netherlands, Austria and Italy, 389 patients with a history of apple allergy were included in the study. Among these patients females dominated. This has been observed in many other studies but an explanation has so far not been proposed. Whether it is related to genetic differences or to behavioral differences still needs to be investigated. The age distribution showed striking differences as well. Patients in Spain were significantly younger than those in NL and A, whereas those in Italy were significantly older. It can not be completely ruled out that these differences are simply explained by the age distribution of the patient populations covered by the clinical centres. The age at onset of apple allergy was significantly lower in Spain than in the other three countries, and coincided with the onset of (grass) pollen allergy. In NL, A and to a lesser extent in I, (birch) pollen allergy started significantly earlier than apple allergy. In Spain, apple allergy the time interval between the onset of pollen allergy and apple allergy was similar to that observed in Italy. The main difference between NL, A and I on the one hand and E on the other hand was the onset of peach allergy. Only in Spain peach allergy started at the same time as pollen allergy. RAST and SPT data demonstrated that IgE reactivity to apple (and Mal

d 1) in NL, A and I was lower than that to birch pollen (and Bet v 1), whereas in Spain it was lower than that to peach. Together, these data confirm earlier reports that apple allergy in areas endemic for birch trees is a result of primary to birch pollen[55,56], whereas in areas without birch trees apple allergy is linked to primary sensitization to peach[49,50]. Non-pollen related food allergies usually occur at younger age than pollen allergies and their related food allergies. If peach consumption is indeed at the basis of apple allergy in Spanish patients, the younger age of the Spanish patients is most likely not caused by a bias introduced by the clinical centres.

Although exposure to birch pollen is virtually absent in Madrid (www.fao.org/faostat), a significant number of patients demonstrated IgE reactivity to birch pollen, Bet v 1 and its homologue in apple Mal d 1. It is highly unlikely that these antibodies were induced by exposure to Bet v 1. This is supported by the observation that IgE reactivity to Mal d 1 was higher than that to Bet v 1. In areas where birch pollen exposure is high, IgE response to Bet v 1 are always higher than to Mal d 1. Possibly, another source of pollen causing exposure to Bet v 1-like allergens is at the basis of Bet v 1 and Mal d 1 sensitization. A candidate might be oak tree pollen[57].

Analysis of IgE responses to individual apple allergens demonstrated that apple allergy in NL, A and I is dominated by Mal d 1, whereas in Spain Mal d 3 is the most important allergen. Apple allergy in Spain has a much more severe clinical presentation than in the other three countries. There is a strong association between severe symptoms and IgE antibodies against Mal d 3 or apple LTP (OR: 7.7). IgE antibodies to Mal d 1 predispose for local symptoms only (OR: 3.3). The explanation for this difference in clinical presentation is the high resistance of LTP against proteolytic attack in the gastro-intestinal tract[28]. Mal d 1 is immediately digested whereas Mal d 3 can reach the gut immune system in an allergenic conformation thus allowing the induction of systemic reactions. The application of purified natural or recombinant major allergens therefore allows distinguishing IgE responses with mild clinical consequences and those with potentially severe and even life-threatening effects. This is a significant step forward for the diagnosis of food allergy compared to the current generation of tests based on food extracts[23].

One question remains open. Why is sensitization to LTP (Mal d 3) not observed in The Netherlands or Austria? Apple consumption is certainly not lower in these countries than in Spain. Perhaps a difference in the consumption rate of peaches can explain the difference. Preliminary analysis of available production and sales data from these countries do not point towards clear differences in consumption levels for apple or peach (data not shown). Reliable consumption data are however not available and are urgently needed. Since peach allergy starts at younger age in Spain, perhaps evaluation of consumption data should be focused at young children. In a small pilot study carried out by the clinical investigators of the four clinical centres, the presence of peach in ready-made meals and drinks for babies and toddlers was analyzed. To this end, products sold in supermarkets in the four centres were screened for the presence of peach. A significant higher chance of finding peach in these products was observed for Spain. In addition, it is not unlikely that fresh peach is given to Spanish children more frequently than to Northern and Central European children. Therefore, perhaps lower exposure in NL and A to peach at young age is the explanation for the lack of sensitization to LTP in these countries. It is of great importance for the development of strategies to decrease the burden of fruit allergies to establish in well-designed controlled studies whether indeed differences in consumption patterns are at the basis of the observed differences in sensitization patterns and clinical presentation.

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3 Allergy to jackfruit: a novel example of Bet v 1-related food allergy

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Allergy 2004: in press

Abstract

Background: Jackfruit allergy has been reported just once. It is unknown whether this food allergy is caused by direct sensitization or cross-sensitization to pollen allergens.
Objective: Establish whether jackfruit allergy is linked to birch pollen allergy.
Methods: Two jackfruit allergic patients and five patients with birch pollen related apple allergy were recruited. Sensitization to pollen and plant foods was assessed by skin prick test (SPT), radio-allergosorbent test (RAST) and immunoblot. RAST analysis was performed for Bet v 1 and Mal d 1. Cross-reactivity was evaluated by RAST and immunoblot-inhibition. Biological activity of IgE was measured by basophil histamine release. Allergy to jackfruit was evaluated by double-blind placebo-controlled food challenge (DBPCFC) or open challenge (OC).
Results: In both patients DBPCFC confirmed the reported jackfruit allergy. SPT was 41 and 27 mm^2 and specific IgE to jackfruit was 5.9 and 0.8 IU/ml, respectively. Immunoblot analysis revealed IgE reactivity at an Mr of approximately 17 kDa. The Bet v 1 related nature of this allergen in jackfruit was demonstrated by RAST and immunoblot inhibition. To assess whether jackfruit allergy might be common in patients with combined birch pollen-fruit allergy, five such patients underwent an OC with jackfruit. All five had oral allergy symptoms.
Conclusions: Jackfruit allergy can be added to the list of birch pollen related food allergies. Increased consumption of this fruit will result in a rise in allergic reactions.

Introduction

IgE-mediated food allergy can be caused by a primary sensitization to pollen inhalant allergens[1,2] or latex[3] resulting in cross-reactive IgE antibodies to a variety of foods. Alternatively, primary sensitization can occur by consumption of food. It is well established that up to 70% of tree-pollen allergic patients from Northern and Central Europe (mainly birch, alder and hazel) display allergic symptoms when eating fruits (e.g. apple, peach and pear) and tree nuts (e.g. hazelnut, walnut)[4]. Some patients also report allergy to vegetables like carrot or celery[5-7]. This is mainly due to cross-reactivity between the major birch pollen allergen, Bet v 1, and its homologues in fruits, nuts and vegetables. In addition, the ubiquitous protein profilin[8,9] and cross-reactive carbohydrate determinants (CCDs)[10-12] have been implicated in cross-reactivity between pollen and plant foods[13,14]. Symptoms in patients with pollen-plant food cross-reactivity are generally mild and limited to the oral cavity, the so called oral allergy symptoms (OAS)[15]. It is characterized by immediate itching in mouth and throat and is sometimes associated with mild to moderate angioedema. Some patients also develop rhinoconjunctivitis and mild asthmatic symptoms[16]. More recently, birch pollen related allergens in carrot and celery and Bet v 1 homologues in soy have been implicated in more severe allergic symptoms as well[17,18].

In this study we describe two birch pollen allergic patients with a relatively severe allergic reaction to fresh jackfruit. Jackfruit (Nangka, *Artocarpus integrifolia)* a tropical fruit belonging to the Moraceae family (mulberry) and to the genus Artocarpus (breadfruit tree) is native to India, East Asia, South-America and East-Africa. Allergy to jackfruit has been described once before for a single patient[19]. In this study, cross-reactivity to birch pollen allergens was not detected. The aim of our study was to confirm jackfruit allergy by double-blind placebo-controlled food challenge (DBPCFC) and to investigate whether crossreactivity with birch pollen is at the basis of the reported allergic reactions to jackfruit.

Material and Methods

Patients

Two adult patients visited the outpatient department of the University Medical Center Utrecht, reporting a severe reaction to jackfruit. Both patients claimed they had never eaten jackfruit before.

Five typical birch pollen and apple allergic patients of the University Medical Center Utrecht were recruited for open food challenges with jackfruit. None of them had ever eaten jackfruit prior to the open challenge. All patients had seasonal rhinitis in the period from March to May (birch pollen season in The Netherlands) and had a positive skin test (> 7 mm^2) and a positive RAST (> 0.7 kU/ml) for birch pollen and apple. The study was reviewed and approved by the local ethical committee. Informed consent was obtained from all subjects before enrolment in the study.

Skin Prick Tests

SPT were performed on the flexor aspect of the forearm with a standardized prick needle (ALK Lancet) and documented according to Dreborg[20]. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT-extracts was used as a negative control (ALK- ABELLÓ, Nieuwegein, The Netherlands). Patients underwent SPT with commercial extracts from birch, grass, olive, mugwort, parietaria and ragweed pollen and from celery, hazelnut, peanut and walnut (ALK-ABELLÓ). SPT for fresh apple, jackfruit and peach were performed with fresh fruits using the prick-to-prick method. The wheal reaction was measured after 15 minutes and transferred with transparent adhesive tape on to a record sheet. The skin wheal areas were determined by computer scanning[21]. SPT were regarded positive when the wheal area was at least 7 mm^2.

DBPCFC/Open challenges

Clinical reactivity to jackfruit was investigated by DBPCFC in the two birch pollen allergic patients that had reported an adverse reaction to this food. DBPCFC was carried out using a procedure, which has been described elsewhere[22].

The challenge meals were prepared within five minutes before administration and contained: 5 and 10 grams of fresh shredded jackfruit, respectively. The meals were completed with a mixture of yoghurt, orange juice, apple juice, applesauce and oatmeal flakes. The placebo doses consisted of the same ingredients except fresh jackfruit. Apple juice and sauce do not contain any IgE-reactive Mal d 1 allergen (Bet v 1 homologue) due to processing steps and were added for optimal blinding of taste. In addition the patients were nose-clipped to mask odor and taste. The patients were under continuous observation during the challenge test and all symptoms were recorded.

Open challenges were performed with 2 samples (5 and 10 gr) of fresh jackfruit. The same method of scoring was used and the same precautions were taken as in case of the DBPCFC.

Extracts and allergens

Jackfruit and apple were obtained from a local food store. Extracts were prepared essentially as described by Björksten *et al.*[23]. In brief, the fruit (without peel and core) was homogenized at 10% w/v in 0.1 M phosphate buffer pH 7.0, containing 2% polyvinylpolypyrrolidone, 7 mM diethyldithiocarbamate, 2 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and 2.6 mM NaN3. After stirring for 1 hour, particulate matter was removed by centrifugation at 18,000 g for 30 minutes. The supernatant was dialyzed against distilled water and lyophilized. Birch pollen extract was made according to the protocol described by van Ree *et al.*[24]. rBet v 1 and rBet v 2 were purchased from Biomay (Vienna, Austria), nBet v 1 and nMal d 1 were purified by affinity chromatography using monoclonal antibody (mAb) 5H8 (directed to nBet v 1 and cross-reactive to several plant food homologues)[25]. For application in RAST, 4 mg jackfruit or apple protein or 100 μg purified protein were coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham-Pharmacia-Biotech, Uppsala, Sweden).

RAST and RAST-inhibition

RAST was performed as described previously[26]. For RAST inhibition, serum was pre-incubated with (serial) dilutions (in 50 µl PBS-AT) of inhibitor (birch pollen or jackfruit extract or nBet v 1), prior to addition of Sepharose-coupled jackfruit or birch extract. For the uninhibited value, serum was pre-incubated with 50 µl PBS-AT. Subsequent steps were identical to those described for the RAST. Results were either expressed as percentage inhibition, or as IU/ml.

SDS-PAGE/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, California, USA) and silver stained using a protein silver staining kit (Bexel Biotechnology, Union City, CA, USA).

Western blotting was performed by transferring the proteins semi-dry to 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 minutes, the blots were cut into strips prior to immunoprobing overnight with 150 µl human serum in 3 ml of PBS-AT. After washing away unbound serum with PBS/ 0.1% Tween-20, radiolabeled sheep antibodies against human IgE were used for detection of bound IgE. Blots were exposed to X-ray film (Eastman Kodac Company, Rochester, NY, USA). For blot inhibition studies, 150 µl of the inhibitor was added in several concentrations together with the patient serum. Incubation of the blotstrips and detection were performed as above.

Basophil histamine release assay (BHR)

White blood cells were isolated from blood of a non-allergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere[27,28]. Subsequently cells were resensitized with the patients' serum. Histamine release was performed with apple, birch and jackfruit extract (1 ng/ml – 100 µg/ml) and with purified nBet v 1 (100 pg/ml-10µg/ml). Liberated histamine was measured by the fluoretic method essentially as described by Siraganian[29]. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Center under project number: MEC97 / 030.

Results

Case report 1

A 31-year-old man with a history of hay fever in the birch pollen season increasingly reported episodes of OAS after eating apple, hazelnut or peanut. On a holiday in Thailand, he developed OAS within 5 minutes after eating a very small piece of fresh jackfruit. Subsequently, he developed hoarseness, swelling of the throat and dyspnoe 10 minutes later. He had never eaten this fruit before.

Case report 2

A 27-year-old female with hay fever in the birch and grass pollen season reported increased incidence of OAS after eating apple, hazelnut and peanut since early youth. Recently she experienced OAS and abdominal cramps within 5 minutes after eating a small piece of fresh jackfruit for the first time.

Confirmation of jackfruit allergy by SPT, RAST and DBPCFC

For patient 1, prick tests were strongly positive for the jackfruit (41 mm^2) and RAST values (kU/ml) were positive for birch pollen (35.9), apple (21.4) and jackfruit (3.4). An allergy to jackfruit was confirmed by DBPCFC (VAS score: 90). He reacted with oral allergy (itching in mouth and throat) after a relatively small dose (5 g) of jackfruit. No reaction occurred on placebo.

For patient 2, the prick tests were strongly positive for jackfruit (27 mm^2) and RAST values (kU/ml) were positive for birch pollen (3.8), apple (1.6) and jackfruit (0.8). The DBPCFC result was positive for jackfruit (VAS score: 40). The patient reacted with oral allergy (itching in mouth and throat) and abdominal cramps already after 5 g of jackfruit. No placebo reaction.

Clinical histories and skin test results of both patients with reported jackfruit allergy are summarized in table 1. Based on their clinical history, the patients were also tested by RAST for specific IgE to additional foods (Table 2).

Table 1. CASE REPORTS 1-2: Clinical history, skin tests and results DBPCFC

Pt No.	Age (y)	Sex	Rhinoconjunctivitis	Asthma	Food allergy	Positive skin test results (wheal size in mm^2)	Symptoms upon ingestion	DBPCFC	treatment after DBPCFC
1	31	M	Birch and grass	No	ap, pe, ha, jf	Histamine (37), bi (43), gr (47), jf (41), ap (69), pe (76), pea (113), ha (38), ce (48), phl (12), ol (20)	OAS, dyspnoe, hoarseness.	OAS (90 VAS)	Antihistamine
2	27	F	Birch and grass	No	ap, pe, ha, jf	Histamine (24), bi (40), gr (41), jf (27), ap (22), pe (61), pea (16), ha (27), ce (14), phl (20), ol (10)	OAS, cramps.	OAS (40 VAS), cramps	Antihistamine

ap, apple; *bi,* birch pollen; *gr,* grass pollen; *ha,* hazelnut; *jf,* jackfruit; *ol,* olive pollen *pea,* peanut; *pe*, peach; *phl,* phleum pratense. *OAS*, Oral allergy symptoms (Itching and swelling of the lips, mouth, throat); *DBPCFC,* double blind placebo controlled food challenge. (only positive SPT results: > 7 mm^2 are shown)

Table 2. Results of specific IgE determinations cases 1 and 2

Case	1	2
	IU/ml	IU/ml
Phleum	5,6	0,3
Birch	35,9	3,8
Peach	5,6	0,8
Hazelnut	8,8	1,5
Apple	21,4	1,6
CCD	0,8	0,4
Jackfruit	5.9	0,8
nBet v 1	95,1	4,9
nMal d 1	11,1	0,6
rMal d 2	0,1	0,1
nMal d 3	0,1	0,1
rMal d 4	0,2	0,3

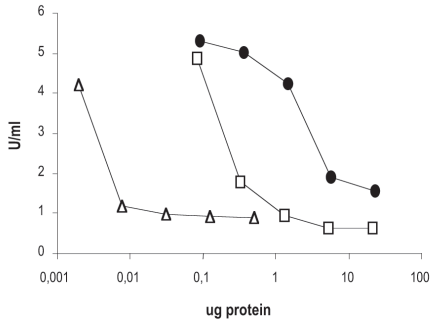
CCD: crossreactive carbohydrate determinants

Jackfruit allergy is related to anti-Bet v 1 IgE

To investigate whether birch pollen related food allergies were indeed caused by Bet v 1 homologues in fruit, serum of both patients was tested on natural Bet v 1 and the four major apple allergens Mal d 1-4 (Table 2). The analysis confirmed the recognition of Bet v 1 related allergens. Binding of IgE antibodies to jackfruit was almost completely (80-85%) inhibited by jackfruit extract, birch pollen extract and nBet v 1. The inhibition with birch pollen extract was stronger (by approximately a factor 25) than with jackfruit indicating birch pollen is most likely the sensitizing agent. Specific IgE to jackfruit of patient 2 was too low to perform RAST inhibition tests (Figure 1).

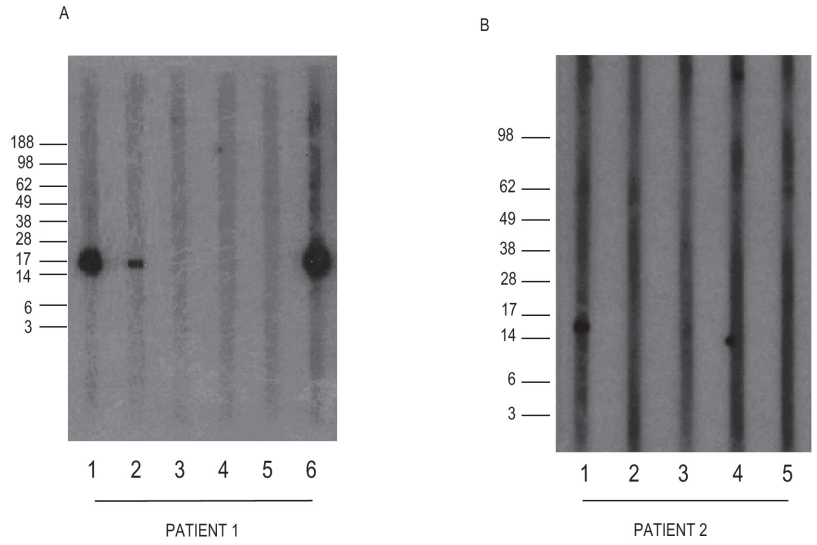
Immunoblot analysis confirmed the presence of a Bet v 1 homologue in jackfruit extract. Use of the monoclonal antibody 5H8 resulted in detection of a 17 kDa protein. Cross-reactivity of jackfruit allergens was further investigated by means of blot inhibition (Figure 2). Both patients recognized a band around 17 kDa. This band was inhibited by addition of nMal d 1 and nBet v 1 and by birch pollen extract (BPE). In addition, for patient 2 the band was completely inhibited by addition of jackfruit extract (not done for patient 1).

Figure 1



RAST inhibition studies with patient 1. Three different extracts were coupled to sepharose and jackfruit extract (circles), birch pollen extract (squares) and nBet v 1 (triangles) were used as inhibitor.

Figure 2



Immunoblot inhibition studies with patient 1 (A) and 2 (B). Lanes 1, uninhibited serum; lanes 2(A) and 4(B), 10 μg/ml Mal d 1; lane 3(A) 2 μg/ml, and lanes 4(A) and 5(B) 10 μg/ml nBet v 1; lanes 4(A) and 2(B) 2 mg/ml birch pollen extract; lane 3(B) 4.4 mg/ml jackfruit extract and lane 6(A) α-Bet v 1 monoclonal 5H8. Marker sizes are indicated on the left hand side.

Histamine release assay

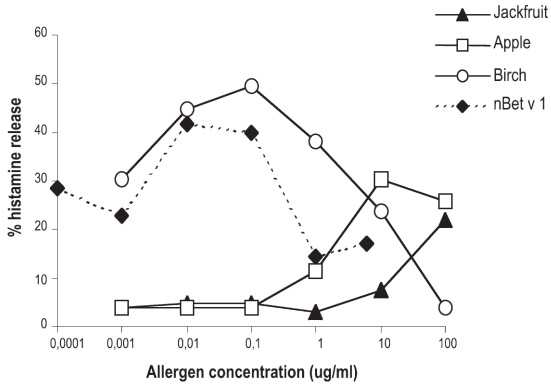
Patients 1 and 2 were tested for their ability to induce histamine release with birch pollen, apple and jackfruit extract and nBet v 1 using the indirect histamine release assay. Patient 1 shows histamine release with all extracts/allergens used and it is clear that lower concentrations of birch pollen extract and nBet v 1 are needed to induce histamine release as compared to apple and jackfruit extract (Figure 3). For patient 2 no histamine release was detected with jackfruit and apple extract, while up to 13% release was detected with birch pollen extract and 37% release with nBet v 1 (results not shown). Stripped cells were used as a negative control and induced a histamine release lower than 3% (data not shown).

Incidence of biologically active anti-jackfruit IgE

Five adult patients (1 male and 4 female, mean age 30.6 years), allergic to birch pollen and apple had a positive skin test (> 7 mm^2) for birch and apple and a positive SPT and RAST to jackfruit. They were subjected to an open challenge with jackfruit. The mean VAS score was 70 ± 15. Three patients reacted with oral allergy (itching in mouth and throat) already after 5 g of jackfruit and the last two patients reacted after 10 g of jackfruit. All five patients had never eaten jackfruit before.

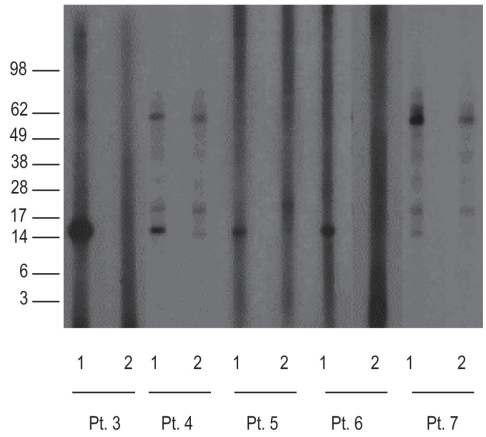
The immunoblot analyses revealed recognition of a 17 kDa protein band, that could be inhibited by addition of nBet v 1 (Figure 4).

Figure 3



Basophil histamine release assay with serum of patient 1. Both apple and jackfruit extract induce a similar response, but birch pollen extract and nBet v 1 induce a higher histamine release.

Figure 4



An immunoblot of jackfruit extract incubated with the different sera. Lane 1: uninhibited serum, lanes 2-5: serum, pre-incubated with 100 μg/ml nBet v 1 (2). Blots of patient 3, 5, 6 have been exposed for a longer time-period. Marker sizes are indicated on the left hand side.

Discussion

Allergy to jackfruit was first reported for a single patient by Wüthrich *et al.*[30]. At that time, a link to birch pollen allergy was not established. In the present study, allergy to jackfruit was for the first time confirmed by DBPCFC. In addition, it was convincingly demonstrated that allergy to jackfruit is a new member of the Bet v 1-related food allergies. Both by RAST-inhibition and by immunoblot and immunoblot-inhibition, the dominant role of cross-reactive IgE antibodies against Bet v 1 for the IgE reactivity to jackfruit was shown.

Despite the high prevalence of sensitization to birch pollen (Bet v 1) in countries of the Northern Hemisphere with a temperate climate, allergy to jackfruit is extremely rare compared to other Bet v 1-related food allergies like allergy to apple and to hazelnut. The main reason for this is the insignificant role of this tropical fruit in the local diets. Open challenges with jackfruit in a group of birch-apple allergic patients that had never consumed jackfruit before, have illustrated that increased consumption of jackfruit will most likely result in a strong increase in the prevalence of allergy to this food. It can be expected that introduction of other new tropical fruits and vegetables will be accompanied by new food allergies.

Both patients in this study reported more severe symptoms in addition to their OAS. One patient complained about having cramps after eating jackfruit. This was confirmed in the DBPCFC. The other patient reported hoarseness and dyspnoe. It is a common generalization that Bet v 1-related food allergies only cause relatively mild symptoms limited to the oral cavity (OAS). This phenomenon has been explained by the lability of Bet v 1-related food allergens in the acidic and proteolytic environment of the gastro-intestinal tract. Recently, allergy to the soy homologue of Bet v 1, Gly m 4, was claimed to cause severe systemic reactions. This report and our observations suggest that some food homologues of Bet v 1 might be more stable than the extremely labile representatives like for example Mal d 1 from apple, enabling them to induce more severe and systemic symptoms. Whether this is the case remains to be determined. When studying stability of food allergens, both the intrinsic properties of the allergen molecules and the possible protective role of the food matrix need to be addressed. Finally, it can not be completely excluded that reported severe symptoms are linked to allergens not (sufficiently) represented in extracts used for RAST- and immunoblot analyses. The poor IgE-binding capacity of jackfruit extract for one of both patients (only 0.8 IU/ml), and the weak capacity to induce histamine release might indeed point in that direction.

In conclusion, allergy to jackfruit can be added to the list of birch pollen-related food allergies. Whether the jackfruit homologue of Bet v 1 is the only cross-reactive structure responsible for the observed clinical symptoms remains to be determined.

Acknowledgements

This study was supported by a grant from the EC: SAFE QLK1-CT-2000-01394.

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4 Severe allergy to sharonfruit caused by birch pollen

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Accepted International Archives of Allergy and Immunology

Abstract

Background: Allergy to sharonfruit (persimmon) has been reported on a few occasions only. Cross-reactivity to pollen on the basis of profilin and Bet v 6 has been shown. Bet v 1 was so far not implicated to play a role.
Objective: To identify whether Bet v 1 sensitization is linked to sharonfruit allergy.
Methods: Two patients with a reaction upon first exposure to sharonfruit were included. In addition, seven patients were recruited on the basis of an established birch pollen related oral allergy syndrome for apple. Sensitization to pollen and plant foods was assessed by skin prick testing (SPT), radio-allergosorbent test (RAST) and immunoblot. RAST analysis was performed for Bet v 1, Bet v 2 and Bet v 6. Cross-reactivity was evaluated by RAST and immunoblot-inhibitions. Biological activity of IgE was measured in basophil histamine release (BHR) tests. Allergy to sharonfruit was evaluated by double-blind placebo-controlled food challenge (DBPCFC) or open challenge (OC).
Results: Both patients who had reported an allergic reaction to sharonfruit had a concordant RAST (8.6 and 6.2 IU/ml, respectively) and SPT (wheal area 37 and 36 mm^2). Sharonfruit allergy was confirmed by DBPCFC in one patient. The second patient refused to undergo a challenge because of the severity of the initial reaction. Both patients had IgE antibodies against Bet v 1 and Bet v 6 that were shown to be cross-reactive to sharonfruit by RAST- and immunoblot inhibition. The patient with severe reactions in addition had a strong IgE response against profilin. Profilin did not induce significant histamine release however, as was the case for Bet v 6. Bet v 1 induced approximately 60% histamine release. An open challenge with sharonfruit in 7 birch pollen- apple allergic patients was positive in 6/7 cases.
Conclusions: Birch pollen-related allergy to sharonfruit is mediated by the known cross-reactive pollen allergens including Bet v 1.

Introduction

IgE mediated food allergy is caused by primary sensitization to pollen[1,2] or latex[3] resulting in cross-reactive IgE antibodies to a variety of foods, or by primary sensitization to food.

In this study, we report two cases of allergy to sharonfruit. Sharonfruit is the edible fruit of the persimmon tree that belongs to the *Ebenaceae* family. Once native to China and Japan, persimmon trees were introduced to Europe and California in the mid-19th century and the fruit is currently produced in California, France, Italy and Spain, where it is known as kaki[4]. A study by Anliker *et al.*[5] on sharonfruit allergy suggested pollen profilin and cross-reactive carbohydrate determinants (CCD) as sources of cross-reactive allergens. In addition, Karamloo and coworkers[6] demonstrated involvement of a Bet v 6-related food allergen (phenylcoumaran benzylic ether reductase, PCBER) as cross-reactive allergen in sharonfruit. So far, the major birch pollen allergen Bet v 1 was not identified as possible cause of cross-reactive sharonfruit allergy.

Bet v 1 accounts for most of the *in vitro* (serologic) and *in vivo* (clinical) cross-reactivity to *Rosaceae* fruits (e.g. apple, peach, pear) and to a lesser extent to *Umbelliferae* vegetables (e.g. celery, carrot, fennel)[7,8]. Food allergy in these patients is generally mild and mostly limited to the oral cavity, the so-called oral allergy syndrome (OAS)[9]. It is generally believed that Bet v 1-related food allergy is mild and limited to the oral cavity, sometimes associated with mild to moderate angioedema. Some patients also develop rhinoconjunctivitis and mild asthmatic symptoms. Due to the fact that food homologs of Bet v 1 do not survive the harsh acidic and proteolytic environment of the gastrointestinal tract. More recently, however Bet v 1-related allergens in carrot and celery and also in soy have been implicated in more severe allergic symptoms as well[10,11]. Although serological observations support a role for these some Bet v 1 homologs in severe symptoms, increased stability has not been proven.

Profilin is a 12- to 15-kDa monomeric actin-binding protein present in all eukaryotic cells. It was first reported as a minor allergen in birch pollen but is an ubiquitous plant panallergen and one of the main causes of cross-sensitization between pollen and plant-derived foods. Sera from patients with pollen allergy sensitized to profilin show IgE cross-reactivity to fruits and vegetables. Profilin has been implicated in the birch-Rosaceae fruit and the birch-mugwort-celery-spice syndrome[12,13] and several studies concluded that this protein can also play a role in patients allergic to a variety of other foods[14-24]. Results of recent studies suggest that IgE antibodies to profilin in foods have little or no clinical re1evance[25-27]. Other publications have clearly demonstrated a close correlation between food allergy and IgE antibodies to profilin. For example, Rodriguez-Perez *et al.* reported a dominant role for profilin in Spanish grass pollen allergic patients with clinical peach allergy[28]. Most likely this discrepancy is caused by selection bias. In the former studies pollen allergy with cross-reactive IgE antibodies was used as selection criterion, but in the latter studies clinical fruit allergy.

More recently Bet v 6 (PCBER) was identified as a cross-reactive allergen between birch pollen and plant foods[29].

The aim of our study was to confirm sharonfruit allergy by double-blind placebo-controlled food challenge (DBPCFC) and to investigate whether birch pollen allergens, in particular Bet v 1 were involved in sharonfruit allergy, in addition to Bet v 2 and Bet v 6.

Material and Methods

Patients

Two adult patients visited the outpatient clinic of the University Medical Center Utrecht, reporting a reaction to sharonfruit upon first ingestion. Seven typical birch pollen and apple allergic patients of the University Medical Center Utrecht were recruited for open food challenges with sharonfruit. None of them had ever eaten sharonfruit prior to the open challenge (OC). All patients were apple allergic and had seasonal rhinitis during the birch pollen season in the Netherlands (March to May) and a positive skin test (> 7 mm^2) and a positive RAST (> 0.7 kU/ml) for birch pollen and apple.

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

Skin Prick Tests

SPTs were performed on the flexor aspect of the forearm with a standardized prick needle (ALK Lancet) and documented according to Dreborg[30]. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT-extracts was used as a negative control (ALK-ABELLÓ, Nieuwegein, the Netherlands). Patients underwent SPT with commercial extract for birch-, grass- and olive pollen, celery, hazelnut, mugwort, parietaria, peanut, ragweed and walnut (ALK-ABELLÓ) and with fresh apple, peach and sharonfruit (prick-to-prick method). Reactions were measured after 15 minutes by copying the wheal reaction with transparent adhesive tape on to a record sheet. The skin wheal areas were determined by computer scanning[31]. SPTs were regarded positive when the area was at least 7 mm^2.

Double-blind placebo-controlled food challenges/ Open challenges

Clinical reactivity to sharonfruit was investigated by DBPCFC in the sharonfruit allergic patients who had reported a mild adverse reaction to this food. DBPCFC was carried out using a procedure, which has been described elsewhere[32]. Due to the known lability of Bet v 1-related food allergens the challenge meals containing 5, 10 and 25 grams of fresh shredded sharonfruit were prepared within five minutes before administration. Open challenges were performed with 2 samples (5 and 10 gr) of fresh sharonfruit. The same method of scoring was used and the same precautions were taken as in case of the DBPCFC.

Extracts and allergens

Sharonfruit was obtained from a local food store. Extracts were prepared essentially according to Björksten et al.[33]. In brief, the fruit (without core) was homogenized at 10% w/v in 0.1 Mol/L phosphate buffer pH 7.0, containing 2% polyvinylpolypyrrolidone, 7 mMol/L diethyldithiocarbamate, 2 mMol/L EDTA and 2.6 mMol/L NaN_3. After stirring for 1 hour, particulate matter was removed by centrifugation at 18,000 g for 30 minutes. The supernatant was dialyzed against distilled water and frozen in aliquots. Other food extracts were prepared

as described elsewhere[34]. Birch pollen extract was made as described before[35]. Recombinant (r)Bet v 2 was purchased from Biomay (Vienna, Austria), natural (n)Bet v 1 and nMal d 1 were purified by affinity purification using monoclonal antibody 5H8 (directed to nBet v 1 and homologs)[36]. rBet v 6 was produced as described elsewhere[37]. For application in RAST, 4 mg extract or 100 μg purified allergen was coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham-Pharmacia-Biotech, Uppsala, Sweden).

Radio-allergosorbent test (RAST) and RAST-inhibition

RAST was performed as described previously[38,39]. Results were expressed as international units IgE per ml (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[40]. A RAST value > 0.3 IU/ml was regarded as positive. For RAST inhibition, serum was pre-incubated with (serial) dilutions (in 50 μl PBS-AT (PBS-Albumin, 0.1 % Tween-20) of inhibitor (nMal d 1, rBet v 2, nBet v 1, rBet v 2, rBet v 6, birch pollen or sharonfruit extracts), prior to addition of Sepharose-coupled sharonfruit extract. For the uninhibited value, serum was pre-incubated with 50 μl PBS-AT. Subsequent steps were identical to those described for the RAST. Results were expressed as percentage inhibition.

SDS-PAGE/Immunoblotting

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

Western blotting was performed by transferring the proteins after separation (semi-dry) to nitrocellulose on a Novablot electrophoretic transfer apparatus, according to the protocol of the manufacturer (Invitrogen). After blocking with PBS-A (phosphate buffered saline, 10 mM EDTA, 0.3% BSA) for a minimum of 10', the blots were cut into strips prior to immunoprobing overnight with 150 μl human serum in 3 ml of PBS-AT. After washing away unbound serum with PBS-T (PBS, 0.1% Tween-20) radiolabeled sheep antibodies against human IgE (Sanquin, Amsterdam, The Netherlands) were used for detection of bound IgE. Blots were exposed to X-ray film (Eastman Kodak Company, Rochester, NY, USA). For blot inhibition studies, 150 μl of the inhibitor was pre-incubated for 30' together with the patient serum. Incubation of the blotstrips and detection were performed as above.

Basophil histamine release (BHR) assay

White blood cells were isolated from blood of a non-allergic donor by Percoll (Amersham Pharmacia Biotech, Upssala, Sweden) centrifugation and stripped from IgE by lactic acid treatment as described elsewhere[41-43]. Subsequently, cells were resensitized with the patients' serum. Histamine release was performed with birch and sharonfruit extract (1 ng/ml – 100 μg/ml and with purified nBet v 1, rBet v 6 or rBet v 2 (100 pg/ml-10μg/ml). Liberated histamine was measured by the fluorometric method essentially as described by Siraganian[44].

Stripped cells were used as a negative control and induced a histamine release lower than 3% (data not shown).

The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Centre under project number: MEC97 / 030.

Results

Case report 1

A 36-year-old female had rhino-conjunctivitis in the birch pollen season since early youth and increasingly experienced OAS after eating apple, banana, hazelnut and peanut (Table 1). After eating sharonfruit for the first time she noted OAS within five minutes. Twenty minutes later she experienced cramps, nausea, itching and erythema of the neck, palms of the hands and feet. Moreover dyspnoea and swelling of the throat occurred. She was hospitalized and treated intravenously with antihistamines and prednisolon.

Case report 2

A 31-year-old man with a history of rhino-conjunctivitis in the birch pollen season increasingly reported episodes of OAS after eating apple, hazelnut and peanut (Table 1). He developed OAS within 5 minutes after eating a very small piece of fresh sharonfruit. He had never eaten this fruit before.

Table 1: Case reports 1 and 2: Clinical history, skin tests and DBPCFC results

Pt No.	Age (y)	Sex	Rhinoconjunctivitis	Asthma	Food allergy	Positive SPT results (wheal size in mm^2)	Symptoms upon ingestion	DBPCFC	Treatment after DBPCFC or after ingestion
1	35	F	Birch and grass	No	ap,pe,ha,ba,sf	Histamine (32), bi (38), sf (36), ap (57), pe (43), pea (21), ha (42), ba (25)	OAS (Itching and swelling of the lips, mouth, throat), dyspnoea, tachycardia, nausea, cramps.	Not willing	Antihistamine and prednisone injections
2	31	M	Birch and grass	No	ap, pea, ha, sf	Histamine (37), bi (43), gr (47), sf (37), ap (69), pe (76), pea (113), ha (38), ce (48), phl (12), ol (20)	OAS	OAS (60 VAS)	Antihistamine

ap, apple; *ba,* banana; *bi,* birch pollen; *sf,* sharonfruit; *ha,* hazelnut;; *ol,* olive pollen; *par*, parietaria; *pe*, peach; *pea*, peanut; *phl*, phleum pratense. *OAS*, Oral allergy symptoms (Itching and swelling of the lips, mouth, throat); *DBPCFC,* double blind placebo controlled food challenge. (only positive SPT results: > 7 mm^2 are shown)

Confirmation of sharonfruit sensitization and allergy by SPT, RAST and DBPCFC

Both patients demonstrated a positive SPT for sharonfruit (36 and 37 mm^2 respectively) and the other fruits and nuts to which reported OAS (Table 1). RAST analysis confirmed sensitization to sharonfruit (8.6 and 6.2 IU/ml, respectively) and the other foods (Table 2). Due to the severity of the initial reaction, patient 1 refused to be challenged. For patient 2, allergy to sharonfruit was confirmed by DBPCFC (Table 1). He reacted with itching in mouth and throat after a relatively small dose (5 g) of sharonfruit. No reactions occurred on placebo.

Table 2: Results of specific IgE determinations

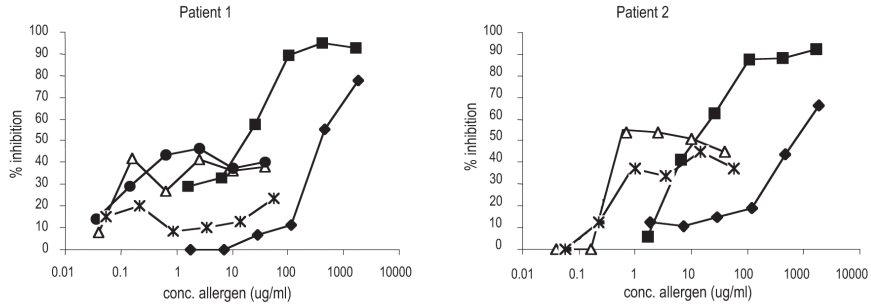
Pt. No.	1	2
	IU/ml	IU/ml
Birch pollen	20.1	35.9
Apple	12.8	21.4
Sharonfruit	8.6	6.16
Peach	9.3	5.6
Hazelnut	11.4	8.8
Peanut	8.7	9.68
nBet v 1	12.5	95.1
rBet v 2	2.7	ND
rBet v 6	1.8	6.1
CCD	0.2	0.8

CCD: Cross-reactive carbohydrate determinants; ND: Not detected

Sharonfruit allergy is caused by IgE against birch pollen allergens

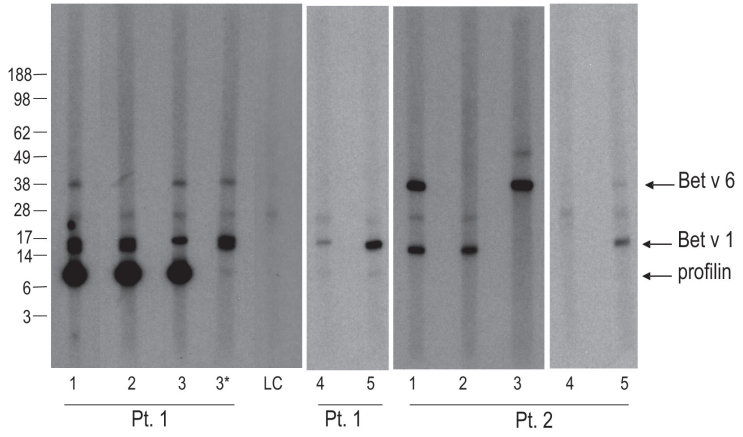
To investigate whether the sharonfruit allergy was caused by allergens homologous to birch pollen allergens, serum of both patients was tested in a RAST on nBet v 1, rBet v 2 and rBet v 6 (Table 2). Both patients recognized nBet v 1 and rBet v 6. Patient 1 also had specific IgE against profilin. RAST inhibition (Figure 1) revealed strong cross-reactivity between birch pollen and sharonfruit. The sharonfruit RAST was inhibited by birch pollen extract (by > 90%) at lower concentrations than by sharonfruit extract, pointing towards birch pollen as primary sensitizer. Partial inhibition was observed with nBet v 1, rBet v 2 and rBet v 6 for patient 1 and with nBet v 1 and rBet v 6 for patient 2. By adding up percentages inhibition achieved with individual allergens complete inhibition was approached. Immunoblot (inhibition) analysis confirmed the presence of the three birch pollen homologs in sharonfruit (Figure 2). Both patients recognized bands around 17 and 38 kDa. The 17 kDa band was inhibited by addition of nBet v 1, the 38 kDa band by rBet v 6. Both bands were inhibited by birch pollen extract (BPE) and sharonfruit extract (SFE). In addition, patient 1 recognized a band around 12-14 kDa, which was inhibited by rBet v 2 and BPE or SFE. Furthermore, this patient recognized an additional band at 18 kDa which was not be inhibited by the addition of nBet v 1, but was partially inhibited by BPE.

Figure 1: Sharonfruit RAST inhibition of patients 1 and 2



Inhibitors: sharonfruit (diamonds), birch pollen extract (squares), nBet v 1 (open triangles), rBet v 6 (asterisks) or rBet v 2 (circles).

Figure 2: Immunoblot inhibition studies with patient 1 and 2

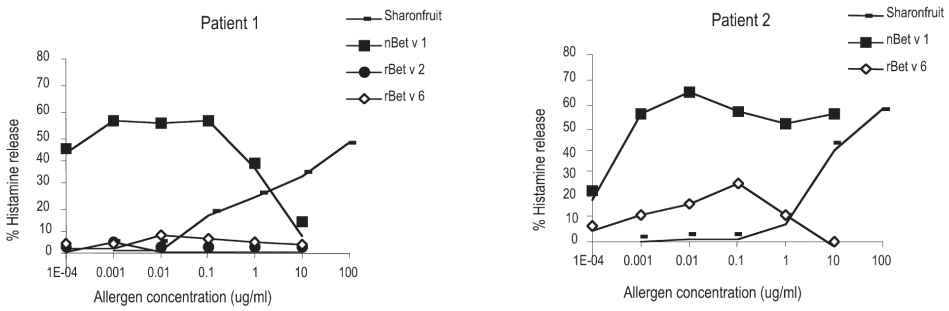


Lanes 1: uninhibited serum, 2: 10 μg/ml rBet v 6, 3: 10 μg/ml nBet v 1, 3*: 10 μg/ml nBet v 2 (Pt. 1), 4: 4.4 mg/ml birch pollen extract, 5: 2.5 mg/ml sharonfruit extract and LC: Labelcontrol (also containing a-specific bands).

Biological activity of cross-reactive IgE

To assess the biological activity of anti-sharonfruit and anti-Bet v 1, 2 and 6 IgE, histamine release tests were performed (Figure 3). Sharonfruit extract induced 45 and 55% release, respectively. Bet v 1 resulted in >50% release at a concentration of 1μg/ml. Bet v 2 and Bet v 6 did not have significant biological activity (<10%) in patient 1. Specific IgE against Bet v 6 had significant but poor biological activity for patient 2, i.e. <25% release at 100 μg/ml.

Figure 3: Basophil histamine release assay with serum of patient 1 or 2



nBet v 1 (closed squares), sharonfruit extract (line), rBet v 6 (open diamonds)and rBet v 2 (patient 1, closed circles).

Sharonfruit allergy in patients with birch pollen apple related allergy

Seven adult patients (patients 3-9, 2 female and 5 male, mean age 35.2 years), with a convincing history of allergy to birch pollen and apple and a positive skin test (> 7 mm^2) for both allergens had a positive SPT to sharonfruit (Table 3). All had significant but relatively low specific IgE titers to sharonfruit with a mean of 0.94 IU/ml ranging from 0.36-1.92 IU/ml. None of them had eaten sharonfruit before. They were subjected to an open challenge with sharonfruit. Six of seven patients reported OAS (itching in mouth and throat) already after ingestion of 5 g sharonfruit.

Table 3: Specific IgE determination, skin test and OC results of patients 3-9

Pt. No.	Birch	Apple	Sharonfruit	SPT (mm^2)	OC
	IU/ml	IU/ml	IU/ml		
3	8.9	3.1	0.5	10	pos
4	4.9	1.1	0.7	36	pos
5	29.8	9.6	1.9	39	pos
6	3.8	1.6	0.4	24	pos
7	9.6	1.3	1.2	29	pos
8	3.3	1.6	1.8	19	pos
9	4.8	0.5	0.6	9	neg

OC: Open challenge, Pos: Positive; Neg: Negative

Discussion

Allergy to sharonfruit was first described by Prandini *et al.*[45] and Martinez *et al.*[46]. The study of Anliker *et al.*[47] suggested a role for primary sensitization by grass pollen. They implicated profilin and CCD as the main allergens in sharonfruit. None of these studies linked sharonfruit allergy explicitly to birch pollen allergens.

In this study, we describe two patients with a birch pollen related food allergy with an adverse reaction to sharonfruit upon first contact with this fruit, which was severe in one case. This observation strongly suggested a role for birch pollen as primary sensitizer. Indeed by RAST- and immunoblot inhibition cross-reactivity between sharonfruit and birch pollen was confirmed. Studying this cross-reactivity more in detail demonstrated a major role for Bet v 1. In agreement with the findings of Anliker and co-workers, profilin appeared also involved in one of the two patients. Furthermore, Bet v 6 was shown to be a cross-reactive allergen for both patients, as was earlier reported by Karamloo[48].

Compared to Bet v 1, IgE titers against profilin and Bet v 6 were much lower. Furthermore, the biological activity of IgE antibodies against both profilin and Bet v 6 was low or negligible, suggesting that these cross-reactive allergens are of limited clinical relevance to these patients. Poor biological activity has been reported for several cross-reactive structures including profilin and CCD. The only allergen that demonstrated clear biological activity was Bet v 1.

Although this study has convincingly shown that Bet v 1 plays an important role in birch pollen - sharonfruit cross-reactivity, its clinical relevance is still not completely clear. In general, Bet v 1 homologs in fruit are thought to be extremely sensitive to gastro-intestinal proteolysis and therefore to induce only mild oral symptoms. However, the serology and biological activity test performed for patient 1 suggest that the sharonfruit homolog of Bet v 1 is instrumental in the patient's severe sharonfruit allergy. Severe food allergy has been reported for some Bet v 1 homologs, like carrot (Dau c 1)[49], celery (Api g 1)[50] and soy, (Gly m 4)[51].

Sharonfruit is a relatively recent fruit on the European market. This study has shown that it can be expected that the number of adverse reactions in birch pollen allergic patients will increase if consumption figures grow. Six out of seven birch pollen allergic patients that did not eat the food previously had a positive open challenge. This is in line with a recent study in Sweden[52] where 397 birch pollen allergic patients were asked to fill in a questionnaire about possible reactions to a number of exotic fruits that are not common in that area and that they had not eaten previously. From the 112 patients that had eaten sharonfruit before, 19 (17 %) reported an allergic reaction to the fruit.

Recently, we reported similar findings for an even less common fruit, i.e. jackfruit[32]. From the regulatory point of view, it is doubtful whether sharonfruit or jackfruit would now still be allowed to enter the market. A recent report[53] on a tropical nut, the nangai nut, suggests that this novel food should perhaps have been refused a marketing authorization on the basis of allergenicity following the EC guidelines on novel foods. Whether allergenicity of a novel food is enough reason to deprive all consumers from access to such foods remains an interesting debate.

In summary, we have demonstrated that allergy to sharonfruit is a new member of the birch pollen related fruit allergies. Bet v 1 plays an important role in serological cross-reactivity,

in contrast to previous studies. The clinical relevance and the relevance contribution of the different allergens involved need further studies.

Acknowledgements

This study was supported by a grant from the EC: SAFE QLK1-CT-2000-01394.

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In vivo assessment by prick-to-prick testing and double-blind placebo-controlled food challenge of allergenicity of apple cultivars

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Submitted

Abstract

Background: Apple cultivars have been reported to differ in their major allergen activity by *in vitro* tests and skin prick tests (SPT) using apple extracts. The major apple allergen Mal d 1 is extremely labile during processing and its IgE-binding potency can easily be lost during extraction e.g. for use in SPT.

Objectives: The aim of this study was to evaluate the efficacy of the prick-to-prick method for assessment of the allergenicity of apple cultivars and to confirm observed differences in allergenicity by double-blind placebo-controlled food challenge (DBPCFC).

Methods: Intra-assay and intra-cultivar variation of prick-to-prick testing were determined in 6 Dutch and 8 Spanish apple-allergic patients using five apples of three cultivars in duplicate. Furthermore, twenty-one cultivars (cold stored for half a year at 2ºC) were screened for potential differences in allergenicity in Dutch birch pollen- and apple-allergic patients. Three cultivars were re-tested one year later to assess reproducibility of ranking. In addition, Golden Delicious, Gala, Santana, Fuji and Braeburn were stored for 6 months with low temperature and without controlled oxygen and carbon dioxide levels to determine the influence of oxygen saturation on allergenicity. Finally, observed differences in allergenicity were evaluated by DBPCFC.

Results: Intra-assay variation of SPT was 3.9% and intra-cultivar variation 4.1%. A ranking of 21 cultivars was made based on prick-to-prick testing in 9 patients. Not all differences between cultivars reached significance. Golden Delicious was found to be high allergenic with 5 rankings in the upper tertile and 4 in the middle. Santana was low allergenic with 7 rankings in the lower tertile, one in the middle and one in the higher. Santana was significantly less allergenic than Gala ($p<0,05$) and Golden Delicious ($p<0.05$). Upon re-testing one year later, this difference between Golden Delicious/Gala and Santana was confirmed. Using the same three plus two extra cultivars it was demonstrated that prolonged storage at 3ºC under controlled atmosphere of 2,5% oxygen and 1% carbon dioxide resulted in a small but significant ($p < 0.001$) reduction in allergenicity (15%) compared to storage under uncontrolled conditions. Finally, the observed difference in allergenicity between Santana and Golden Delicious was confirmed in 5 patients by DBPCFC.

Conclusions: Prick-to-prick testing with fresh apples is a reproducible method of assessing allergenicity. Apples can be identified with low or high allergenicity for the majority of patients. This was confirmed by DBPCFC. Selection of cultivars and control of storage conditions are both viable strategies for prevention of symptoms in apple allergic patients.

Introduction

Apples are among the most common edible fruits. Europe 7-8 billion kilograms are consumed yearly[1]. The six most common cultivars on the market (79% market share) are Golden Delicious, Jonagold, Red Delicious, Gala, Elstar, and Granny Smith[1]. Apples are an important source of vitamins and fibres in the diet of the European population. "An apple a day keeps the doctor away" is a common saying. Unfortunately, up to 2% of the Northern- and Central European population is allergic to apple[2]. Apple allergy in these parts of Europe is mainly encoutered among birch pollen allergic patients[3,4]. This concordance is explained by cross-reactivity of IgE antibodies between birch pollen and apple allergens[5,6]. The main cross-reactive birch pollen allergen is Bet v 1[7]; its homolog in apple is Mal d 1[8]. Mal d 1 is a proteolysis-sensitive allergen[9] and consequently symptoms are predominantly mild and limited to the oral cavity[10,11]. Furthermore, Mal d 1 does not survive most processing steps like cooking or juice making. Thus, only consumption of fresh apples leads to symptoms. Usually patients with apple allergy are also sensitized and (variably) allergic to other fruits, vegetables and nuts on the basis of the same cross-reactive antibodies[6,12]. In Southern Europe apple allergy is less common and not related to birch pollen allergy[13]. The clinical presentation of apple allergy is however more severe. This has been explained by the nature of the major apple allergen in these patients, i.e. the non-specific lipid transfer protein (LTP), designated Mal d 3[14]. LTP has been shown to be especially abundant in the peel of fruits[15,16].

The current management of food allergy is based on the avoidance of the foods involved and if needed rescue medication. Here the impact on quality-of-life is indisputable. Since Bet v 1-related fruit allergies are usually mild, some patients decide to continue eating apples and related fruits and accept the inconvenience. Nevertheless, the majority of patients will avoid offending foods, depriving of important sources of vitamins, minerals and fibres.

Both Mal d 1 and Mal d 3 are members of the family of the pathogenesis related proteins (PR)[17]. PR-proteins can be induced upon pathogen exposure or physical stress like bruising. There are several occasions along the way from apple tree to consumption where such stress can occur: organic versus conventional cultivation (pathogens), the physical act of picking the fruit, transport and storage. All these factors can potentially influence the level of these PR-proteins, i.e. the allergenicity. In addition, differences in allergenicity have been reported between apple cultivars. These observations were largely based on *in vitro* methods such as ELISA and immunoblotting and *in vivo* methods such as SPT with apple extracts and open oral challenges with pieces of apple[18-20]. Apple extracts used for SPT have frequently been shown to have poor sensitivity due to the lability of Mal d 1 upon extraction[21,22].

The aim of this study was to evaluate the usefulness of prick-to-prick SPT with fresh apples and DBPCFC in assessing differences in allergenicity between apple cultivars. In addition, the influence of storage conditions was investigated. Elucidation of factors that influence allergenicity during handling and storage will help to develop improved production processes that may benefit patients. Furthermore, identification of low-allergenic cultivars may in future allow breeders to develop hypoallergenic apples.

Material and Methods

Patients

Fifteen adult patients were recruited from the outpatient clinic of the department of Dermatology / Allergology of the University Medical Center Utrecht (Table 1). They all had birch pollinosis manifesting with rhinoconjunctivitis during the birch pollen season (April and May), as well as a positive SPT to fresh apple of at least half of the diameter of the positive histamine control. For SPT the apple cultivar Golden Delicious was used. All patients had a typical history of apple allergy, with symptoms like itching and mild swelling of the mouth, throat and sometimes rhinoconjunctivitis after eating an apple (oral allergy syndrome, OAS). In 3 of the 15 patients apple allergy was confirmed by DBPCFC.

For assessment of intra-assay and intra-cultivar variation a second group of patients (n=8) was included (Table 1). They were randomly selected at Fundación Hospital Alcorcón (Madrid, Spain) from a group of 40 patients with apple allergy confirmed by SPT and DBPCFC with Golden Delicious.

This study was reviewed and approved by the Ethics Committees of the University Medical Center Utrecht and/of the Fundación Hospital Alcorcón. All patients gave written informed consent before enrolment in the study.

Skin Prick Test

Apple allergic patients were evaluated by SPT, which were performed on the flexor surface of the forearm using the prick-to-prick-technique according to Dreborg[3,23]. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT-extracts was used as a negative control (ALK- ABELLÓ, Nieuwegein, The Netherlands). The wheal reaction was measured after 15 minutes and transferred with transparent adhesive tape on to a record sheet. The skin wheal areas were determined by computer scanning[24].

SPT responses were standardized by dividing the original wheal area of a prick by that obtained for the histamine control. All patients were tested using the same set of apples for each patient. A single prick per apple was performed for each patient, unless stated otherwise. Golden Delicious was always included as reference for high allergenicity.

DBPCFC

Clinical reactivity to the apple cultivars Golden Delicious and Santana was investigated by DBPCFC in five birch pollen allergic patients with allergy to apple. DBPCFC was carried out as described elsewhere[25]. In short, on the day of the challenge only a light breakfast was allowed no later than 2 hours before the first test meal. The challenges were performed in a clinical research setting equipped for resuscitation and monitoring of vital signs. During the challenge the investigator or a nurse continuously 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. An interval of 15 minutes was allowed between each test meal, or as long as was needed for (subjective) symptoms to disappear. Patients were kept for observation for at least one hour after

resolution of symptoms. The severity of the oral allergy symptoms was scored using visual analogue scales (VAS) with a range of 0-100, where 0 is equal to no symptoms and 100 stands for severe symptoms.

To prevent loss of allergenicity upon disruption of apple tissue, challenge meals were prepared within five minutes before administration. They contained 5, 40 and 120 grams of fresh shredded apple of each cultivar, respectively. The meals were completed with a mixture of yoghurt, orange juice, apple juice, applesauce and oatmeal flakes. The placebo doses consisted of the same ingredients with the exception of fresh apple. Apple juice and applesauce do not contain any IgE-reactive Mal d 1 allergen (Bet v 1 homolog) due to processing and were added for optimal blinding of taste. In addition patients were nose-clipped to mask odour and taste.

Table 1. RAST results (Mal d 1 – Mal d 4); NL: Dutch patients, E: Spanish patients.

Pt nr	Intracultivar variation	Country	nMal d 1	nMal d 2	nMal d 3	rMal d 4
1	Yes	NL	0.4	0.2	0.1	0.3
2	Yes	NL	0.6	0.2	0.1	0.2
3	Yes	NL	0.6	1.0	0.3	2.8
4	Yes	NL	1.7	0.6	0.7	1.1
5	Yes	NL	0.6	0.1	0.1	0.3
6	Yes	NL	0.7	0.2	0.1	0.2
7		NL	0.6	0.1	0.1	0.2
8		NL	1.0	0.1	0.1	0.6
9		NL	2.0	0.1	0.1	0.2
10		NL	1.1	0.1	0.1	0.2
11		NL	2.1	0.1	0.1	0.3
12		NL	1.0	0.1	0.1	0.2
13		NL	1.0	0.1	0.1	0.2
14		NL	5.2	0.2	0.2	0.2
15		NL	2.2	0.1	0.1	0.2
16	Yes	E	0.1	0.1	1.0	0.3
17	Yes	E	0.1	0.3	5.6	7.4
18	Yes	E	0.1	0.2	1.7	0.2
19	Yes	E	0.1	0.1	1.4	0.2
20	Yes	E	1.1	0.1	0.1	4.4
21	Yes	E	0.3	0.3	0.7	0.2
22	Yes	E	0.7	0.3	3.5	0.7
24	Yes	E	0.5	0.5	8.9	0.7

Apple Cultivars

Nineteen different commercially available apple cultivars as well as two experimental breeding selections were examined. All cultivars were grown at the research orchard of Plant Research International, Wageningen, The Netherlands. All fruits were harvested at their usual degree of ripeness for consumption. Some of the harvested fruit was stored in a single cold room at 2°C under uncontrolled atmosphere conditions. In addition, six of the cultivars were also stored at 3°C under controlled oxygen 2,5% and carbon dioxide 1% conditions (4%).

Statistical analysis

Statistical analysis were carried out on log-transformed SPT values. The log-transformation was chosen in order to obtain observations with an approximate constant variance; the variance of the SPT values is approximately proportional to the mean. For the log-transformed SPT, a value of zero corresponds with the reaction to histamine. Values smaller than zero indicate a less severe reaction than the reaction to histamine. In all situations analysis of variance (ANOVA) was carried out using the computer package Genstat. All paired comparisons were made using a two-sided t-test.

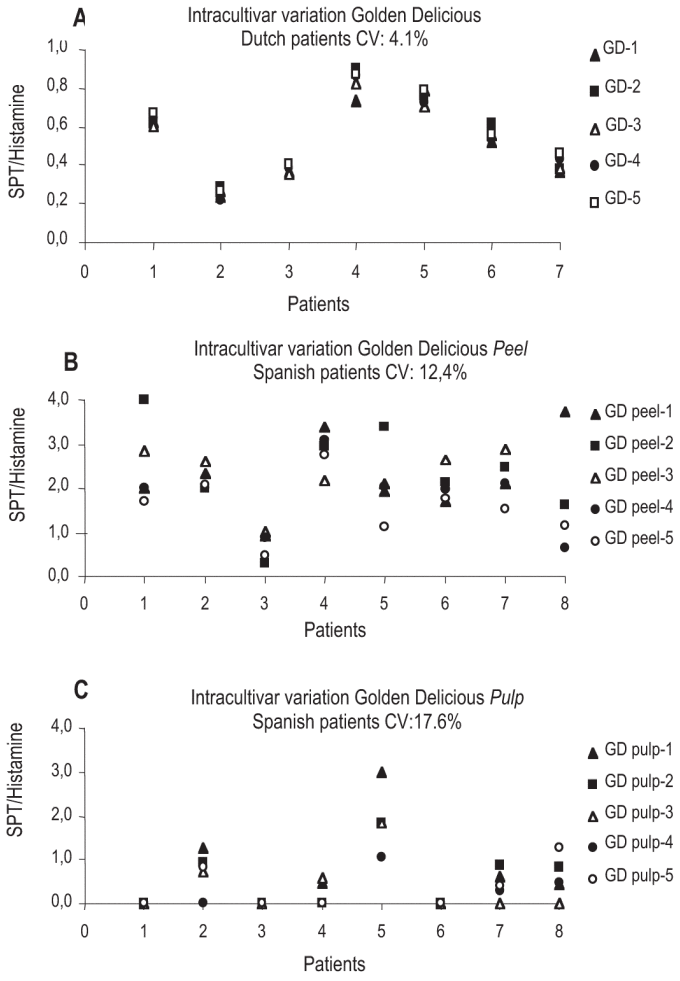
Results

Intra-assay and intra-cultivar variation

To assess the reproducibility of the prick-to-prick method for the determination of allergenicity of apples, 6 out of 15 Dutch patients with established birch pollen-related apple allergy and 8 Spanish patients with non-birch pollen-related apple allergy were included in the study. All patients were tested with 5 apples from each of the following three cultivars: Golden Delicious, Fuji and Ecolette. For each apple two prick-to-prick tests were done per patient. For the Spanish patients, peel and pulp were tested separately for each apple. The intra-assay coefficient of variation among the Dutch patients was 3.9%, among the Spanish patients (peel) 11.5%, (pulp) 14.5%. Intra-cultivar coefficient variations were similar to the intra-assay coefficient of variation, i.e. 4.1% for Dutch patients (Figure 1A) and 12.4% for Spanish (peel) (Figure 1B). This shows that differences between apples of the same cultivar are very small. For the Spanish patients, the test based on the pulp resulted in coefficient variation of 17.6% (Figure 1C).

In the Dutch patients, skin-reactivity to Golden Delicious was significantly higher than to Ecolette and Fuji. For Spanish patients peel, Fuji was more allergenic than Golden Delicious. Ecolette was the least allergenic. These differences were all significant.

Figure 1A B C



6 Dutch and 8 Spanish apple allergic patients using five apples of three cultivars. Intracultivar variation: Dutch patient: CV 4.1%; Spanish patients peel 12.4%; Spanish patients pulp: 17.6%. CV: coefficient of variation

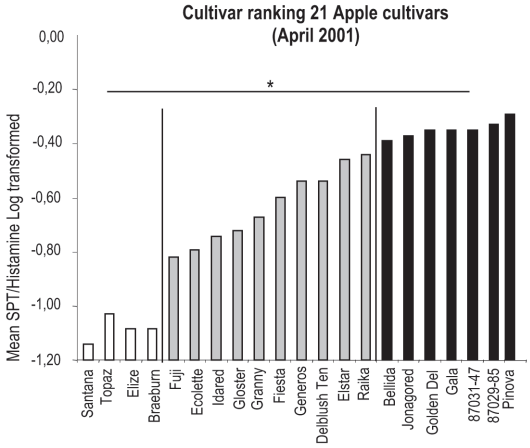
Cultivar ranking

Twenty-one cultivars (Figure 2) were selected for a broader assessment of allergenicity. The cultivars were tested on a single day by the prick-to-prick method in 9 allergic patients. Figure 2 shows a mean ranking of the cultivars of 9 patients with significant differences ($p < 0.05$) in allergenicity between apples of the higher (n=7 cultivars) and the lower (n=4 cultivars) categories, which allows designation of low- (Golden Delicious) and high- (Santana) allergenic apples.

The individual (per patient) rankings of allergenicity were determined and in five patients, Golden Delicious was ranked in the high tertile and in four patients in the middle tertile. For further analyses, the cultivar with the lowest ranking and one cultivar with also a high ranking were selected (next to Golden Delicious): Santana (seven in low, one in middle and one in high tertile), and Gala (two in low, two in middle and 5 in high tertile), respectively.

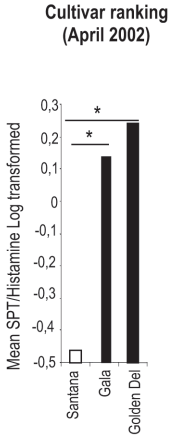
To assess the reproducibility of ranking, Golden Delicious, Gala and Santana were retested one year later. Again these apples had been stored for half year at 2°C. Prick-to-prick testing was performed in six patients, five of whom had participated the year before. This testing resulted in almost the same ranking: Golden Delicious showed the highest mean ranking followed by Gala. Santana again demonstrated the lowest allergenicity (Figure 3).

Figure 2



Ranking of 21 cultivars of 9 Dutch apple allergic patients tested in April 2001. Three categories :Low are low allergenic (white bars), intermediate are moderate allergenic (grey bars) and high are high allergenic cultivars (black bars). The low allergenic cultivars are significantly ($p<0.001$) different of the high allergenic cultivars.

Figure 3

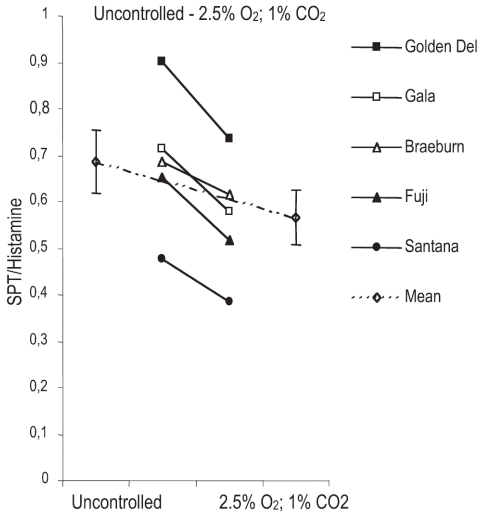


Ranking of 3 cultivars of 4 Dutch apple allergic patients tested in April 2002. White bar: low allergenic; black bars: high allergenic cultivars. Between the high allergenic and low allergenic cultivars it is significantly different ($p<0.001$).

Effect of low-oxygen on allergenicity

In addition to three already tested cultivars (Golden Delicious, Gala, Santana) Braeburn and Fuji were included in this experiment. All cultivars were stored for six months at 2°C under normal oxygen conditions or at 3°C under controlled oxygen (2,5%) and carbon dioxide (1%) conditions. When stored under controlled atmosphere, for all cultivars a reduction of allergenicity was observed by 15%, but however significance ($p<0.001$) was only reached when all cultivars were analysed together (Figure 4). Ranking for allergenicity did not change significantly under controlled oxygen conditions, although two cultivars (Gala and Braeburn) changed places.

Figure 4

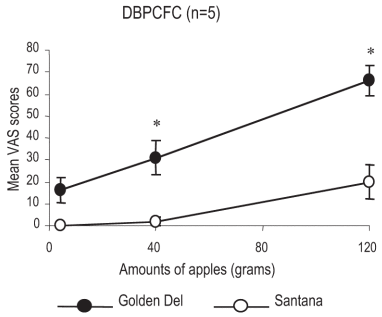


Effect of storage conditions; comparison of cold storage under normal conditions to controlled atmosphere conditions (2,5% oxygen and 1% carbon dioxide). 15 % ($p<0.001$) reduced allergenicity between the storage conditions.

Confirmation by DBPCFC of differences in allergenicity

To confirm the observed differences in allergenicity by the prick-to-prick method, Golden Delicious and Santana were used for DBPCFC in 5 patients. In all these patients SPT for Santana were significantly lower than for Golden Delicious ($p=0.023$). By DBPCFC it was confirmed that Santana indeed was less allergenic than Golden Delicious. The quantities needed for similar VAS scores were on average 30 times higher ($p < 0.001$) for Santana than for Golden Delicious (Figure 5).

Figure 5



The ranking of allergenicity was confirmed by DBPCFC for the highest (Golden Del) and lowest (Santana) cultivars. Results are shown as mean values ± SEM for n=5 patients: Golden Delicious (open circles) and Santana (solid circles). * : $p<0.05$.

Discussion

The allergenicity of different apple cultivars has so far mainly been evaluated by means of *in vitro* techniques[18-20]. In some cases, apple extracts were used for assessment by SPT and in one study clinical evaluation was done by open oral challenge[18]. Although precautions were taken to avoid breakdown of labile allergens like Mal d 1, it is difficult to guarantee complete preservation of allergenicity. Decades ago, Björksten *et al*. described a method of preventing loss of IgE-binding potency during extraction of apples[21]. Adding polyvinylpolypyrrolidone (PVPP) and Na-diethydithiocarbamate (DIECA) inhibited activity of polyphenoloxidases, peroxidases and proteases. This method is still widely used for the extraction of plant foods, in particular of fruits. Others have used a low-temperature acetone method with and without addition of enzyme inhibitors[22,26]. Although these methods are a major improvement in the preparation of apple extracts for diagnostic purposes, not all the components used, as for instance enzyme inhibitors are compatible with *in vivo* use. In addition, Mal d 1 reactivity may still be affected. In a recent *in vitro* comparison of more than 75 different European apple cultivars carried out at two different laboratories, results suggested that extracts from one laboratory had lost a significant part of their Mal d 1 activity despite the fact that both laboratories used an identical protocol with PVPP, DIECA and a cocktail of inhibitors covering a broad spectrum of proteases (manuscript in preparation). These results indicate that small (as yet unidentified) differences in extraction conditions can have a very significant impact. To avoid these problems, we decided to use the prick-to-prick method to assess the allergenicity of apple cultivars. Of course there were serious concerns as to whether this *in vivo* test would be reproducible. Variability was expected to come from differences in pricking the needle into the apple and subsequently into the skin[27]. In other words, is it possible to control the amount of apple allergen applied to the skin? In addition, variability between different apples from the same cultivar might influence the assessment of allergenicity. Finally, variation in skin reactivity within an individual is known to occur. For this reason, all skin reactions were expressed relative to the reactions observed with histamine. To our surprise, both intra-assay variability and intra-cultivar variability were below 5% when tested in Dutch apple allergic patients. Compared to intra-assay variabilities reported for *in vitro* tests like ELISA this was surprisingly good. When tested in Spanish patients, both intra-assay

and intra-cultivar variability for peel increased significantly to values around 12% and values around 18% when pulp was used. The most likely explanation for the difference between Dutch and Spanish patients in this analysis relates to the major allergen that is involved: in Dutch patients Mal d 1, in Spanish patients Mal d 3 (LTP). It has been reported that LTP is mainly expressed in the peel of apples, whereas Mal d 1 is more homogeneously expressed throughout the fruit[15]. It is to be expected that variation in the depth of pricking into the skin of an apple, influences the quantity of Mal d 3 applied to the skin. For Mal d 1 this will have little or no effect. The different ranking of apple cultivars obtained with Dutch and Spanish patients are also explained by the different allergens recognized. This illustrates that allergenicity determined with apple allergic patients from Central or Northern Europe has little or no relevance for patients from Mediterranean countries like Spain.

In our study, we also performed prick-to-prick tests on freshly picked apples in October/November (not shown). It is not really known whether the allergenicity of apples increases during storage. When we compared skin reactivity of cultivars tested immediately after picking with that of the same cultivars stored for six months, a clear increase was observed for most cultivars. Unfortunately, testing after a half year of storage takes place in April/May, at the peak of birch pollen exposure. It is well-known that specific IgE antibody titers rise during birch pollen season[28,29]. Part (if not all) of the increase in skin reactivity observed for stored apples will therefore be caused by increased IgE titers. Therefore, whether storage results in increased allergenicity still needs to be further evaluated. For a reliable analysis, stored apples will have to be evaluated in the skin before IgE titers go up as a consequence of tree pollen exposure in late winter and early spring.

What we were able to establish is that prolonged cold storage in a controlled atmosphere (2,5% oxigen and 1% carbon dioxide) resulted in significantly lower allergenicity than when the atmosphere was not controlled. Although the reduction was moderate (15%), this observation suggests that it makes sense to further manipulating transport and storage conditions as a method of reducing allergenicity in apples.

The ranking of 21 apple cultivars presented in this study has to be interpreted as a first indication. Some of the observed differences did not reach significance because the numbers of patients tested were too small. Nevertheless, the study has provided tools for reliable assessment of allergenicity of apples. Differences in allergenicity between apples of the higher and the lower categories were significant, which allows designation of low- and high-allergenic apples. The reproducible and significant difference in allergenicity between two representatives of these categories, Golden Delicious and Santana, was confirmed by DBPCFC. Although a real threshold dose was not determined, the average 30-fold increase in the dose needed for similar VAS scores suggests that apple allergic patients can eat approximately 30 times more Santana than Golden Delicious before they experience symptoms. This knowledge may offer apple allergic patients a safer way to reintroduce apple into their diets.

Finally, the identification of apples with low allergenicity in combination with increased know-how on the genetic map for Mal d 1 expression may allow breeding of novel cultivars with decreased allergenicity.

In summary, this study has provided support for the usefulness of the prick-to-prick SPT and the DBPCFC in evaluating the allergenicity of fruits. Moreover, these techniques have produced evidence for the existence of significant in vivo differences in allergenicity between apple cultivars that can moreover be influenced by storage conditions. These findings will help to improve food allergy prevention strategies.

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6

Silencing the major apple allergen Mal d 1 using the RNA interference approach

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Submitted

Abstract

Background: Apple allergy is dominated by IgE antibodies against Mal d 1 in areas where birch pollen is endemic. Apples with significantly decreased levels of Mal d 1 would allow the majority of patients in these areas to eat apples without allergic reactions.
Objectives: The aim of this study was to inhibit the expression of Mal d 1 in apple plants by RNA interference (RNAi).
Methods: *In vitro* grown apple plantlets were transformed with a construct coding for a hairpin RNA duplex containing a Mal d 1-specific sense and antisense sequence separated by a Mal d 1-specific intron sequence. Presence of the constructs in transformants was checked by PCR. Expression of Mal d 1 in leaves was monitored by prick-to-prick skin testing in three apple allergic patients and by immunoblotting with a Mal d 1-reactive monoclonal antibody and with IgE antibodies against Mal d 1.
Results: After transformation, plantlets were selected on the basis of having a normal phenotype and growth rate. Using PCR, in six of nine selected plantlets the presence of the gene-silencing construct was demonstrated. By SPT it was shown that a wild type (WT) plantlet had significantly ($p<0.05$) higher allergenicity than five of the transformants. Reduction of expression of Mal d 1 was confirmed by immunoblotting. In WT as well as in the unsuccessful transformants, a strong band was detected with Mal d 1-reactive monoclonal antibody 5H8 at the expected apparent M_r of 17 kDa. This band was virtually absent in the transformants that carried the gene-silencing construct. With human IgE antibodies the same observations were made.
Conclusions: Mal d 1 expression was successfully reduced by RNAi. This translated in significantly reduced *in vivo* allergenicity. These observations support the feasibility of the production of hypoallergenic apples by gene-silencing.

Introduction

Apple allergy is a common phenomenon in patients with birch pollen allergy. The explanation for this concordance is IgE cross-reactivity[1-3]. Around 90% of birch pollen allergic patients have IgE antibodies against the birch pollen allergen Bet v 1[4]. This allergen belongs to a group of so-called pathogenesis-related (PR) proteins, more specifically the PR10 proteins[5]. Many plant foods, in particular fruits and tree nuts, contain homologous proteins that are recognized by the same Bet v 1-specific IgE antibodies. In apple this allergen was designated Mal d 1[6]. Cross-reactive IgE antibody responses against this allergen dominate the IgE response against apple in birch pollen allergic patients[7]. Around 70% of birch pollen allergic patients have been reported to have adverse reactions to apple as a consequence of these cross-reactive IgE antibodies[8]. Although birch pollen-related apple allergy is almost exclusively mild and restricted to the oral cavity, most apple allergic patients avoid the fruit in their diet. Related fruits of the *Rosaceae* family like pear, cherry and peach, as well as tree nuts like hazelnut, can also induce adverse reactions on the basis of the same cross-reactive IgE antibodies[9,10]. Therefore, avoidance often results in deprivation of the diet of a wide range of common plant foods that have important nutritional value.

Apple allergic patients sometimes report that they better tolerate one apple cultivar than another. Differences in allergenicity indeed have been demonstrated[11-13]. Recently, we screened a panel of apple cultivars by the prick-to-prick method and confirmed differences in allergenicity by double-blind placebo-controlled food challenges (DBPCFC) (Bolhaar *et al.*, submitted). Golden Delicious proved to be among the cultivars with high allergenicity whereas Santana was identified as a low-allergenic cultivar. The difference in DBPCFC was around 30-fold. Still the equivalent of approximately half a Santana apple induced a clear adverse reaction. Perhaps conventional breeding strategies can in the future lead to the production of apple cultivars of even lower allergenicity that will allow normal consumption of apples by apple allergic patients.

Over the past decade, progress made in the genetic modification of plants has opened new avenues for the development of hypoallergenic foods. One way to use genetic modification is to introduce mutations into the gene(s) encoding for the major apple allergen Mal d 1 in order to reduce its allergenicity[14]. The three-dimensional structure of Bet v 1 has recently been solved[15] and naturally occurring isoforms and mutants with decreased allergenicity have been characterized[16]. Surface-exposed residues that play a critical role in antibody binding were identified[17-19]. On the basis of this information a mutant of Mal d 1 with five point mutations was designed and expressed in *E. coli* (Ma *et al.*, Manuscript submitted). This mutant molecule was shown to have significantly decreased allergenicity in skin prick testing (SPT) and DBPCFC (Bolhaar *et al.*, submitted). On the basis of these findings, transgenic mutant apple plants were designed. One of the problems of such an approach is that a hypoallergenic mutant is not hypoallergenic for every patient. This has been reported for mutants of major peanut allergens[20-22]. Another problem is the fact that Mal d 1 represents an extended gene family of at least 18 members (Gao *et al.*, personal communication). Their individual level of expression in the fruit and their allergenicity has not yet been elucidated. This makes designing a hypoallergenic apple plant a complex matter. Therefore, the production of an apple plant with significant reduction of the expression level of Mal d

1 would be an attractive alternative because its effect is independent of the individual Mal d 1 genes and antibody specificities.

Recently, several techniques have been developed to silence specific genes in (crop) plants. Such techniques have been applied to genes encoding for allergenic proteins in rice[23] and soy[24]. One of the more recent approaches used to silence genes is RNA interference (RNAi)[25]. This method for post-transcriptional silencing of specific genes (PTGS) aims at sequence-specific RNA degradation. It appears to be highly efficient, especially when a gene construct is used that encodes intron-spliced RNA and forms a hairpin structure[26]. Basically, such a construct is built-up from a sense and an antisense arm with homology to the target gene. These sequences are separated by an intron sequence. The self-complementary (sense and antisense) arms hybridize and, together with the intron sequence, form an RNA duplex (hairpin). Endogenous mRNA seems to be a target of (ds)RNA-mediated genetic interference. It is proposed that RNAi works by double-stranded (ds)RNA-directed, enzymatic RNA degradation[27]. In this way, the endogenous RNA is prevented from passing from the nucleus to the ribosomes where it normally directs protein production. In apple, advantageously, representatives of the Mal d 1 gene family contain a single intron or are intron-less (NCBI database; Gao *et al.*, personal communication). One such intron-containing Mal d 1 gene (from the cultivar Gala) has been isolated to build the construct for PTGS of Mal d 1. In the present paper, RNAi is used to silence genes for Mal d 1.

Normally it takes around five years to grow trees that produce fruits. To be able to evaluate at an earlier stage whether silencing was effective, Mal d 1 expression was measured in leaves of young apple shoots growing in vitro. This study has provided support for the feasibility of producing hypoallergenic apples by silencing the genes for Mal d 1.

Materials and Methods

Plant cultures

For transformation, the apple cultivar Elstar (E94) was used. This cultivar was grown under *in vitro* conditions on medium containing MS (4.6 g/l)[28], sucrose (30 g/l), BAP (0.7 mg/l), FeDDHA (96 mg/l), and Daishin agar (9g/l). For transformation, the *in vitro* leaflets were subdivided into four explants. After the transformation, the explants were cultured on the following media, in order of use:

Shoot induction medium (SIM): MS (4.6 g/l), sorbitol (30 g/l), TDZ (8.8 mg/l), NAA (0.1 mg/l), kanamycin (150 mg/l) cefotaxim (250 mg/l) and phytagel (3 g/l);

Shoot elongation medium (SEM): MS (4.6 g/l), galactose (10 g/l), sucrose (20 g/l), BAP (1 mg/l), GA3 (0.1 mg/l), cefotaxim (250 mg/l) and Daishin agar (9 g/l);

Shoot propagation medium (SPM): MS (4.6 g/l), sucrose (30 g/l), BAP (0.7 mg/l), FeDDHA (96 mg/l), Daishin agar (9g/l).

Before autoclaving, all the media were adjusted to pH 5.7.

Construct for gene-silencing

For gene-silencing, the hairpin design of Smith *et al.*[26] was chosen. The presence of a single endogenous intron in the Mal d 1 gene is advantageous, because such sequences were

reported to increase the gene-silencing effect of the construct significantly. The design of a set of suitable primers was based on a published sequence of Mal d 1 that was designated as Mal d 1b by Son *et al.*[13] (AF020542). The methods involved the following successive steps (see also Figure 1 and Table 1; restriction sites in the primers used for the cloning procedure are given in bold):

The two fragments for synthesis of the hairpin construct were obtained by PCR from genomic DNA isolated from the cultivar Gala. For Fragment 1, the 5'-end primer 1 (All1HpaI) and the 3'-end primer 2 (All2SmaI) were used, resulting in a 516 bp fragment encompassing the 5'UTR, Exon1, Intron and a small part of Exon2. For Fragment 2 the 5'-end primer 1 (All1HpaI) and the 3'-end primer 3 (All3SmaI) were used, resulting in a 276 bp fragment encompassing the 5'UTR and Exon1. Primers All1HpaI and All2SmaI were designed based on the sequence of the Mal d 1 gene obtained from the DBGBT (AF020542) (Data Base: GeneBank-today, GenomeNet, 2000). Primer All3SmaIwas designed based on the sequence of Fragment 1.

For amplification, the DNA was denatured at 96 ºC for 2 min and subjected to 40 cycles of PCR using Pwo polymerase (Roche Diagnostics). Each PCR cycle consisted of denaturation at 96 ºC for 30 sec., annealing at 60 ºC for 30 sec., and primer extension at 72 ºC for 45 sec. The amplified fragments were purified from agarose gel, digested and cloned in the pRAP 37 expression cassette (Florack, unpublished) and verified by sequencing. The pRAP 37 expression cassette contains a Cauliflower mosaic virus 35 S promoter with a double enhancer sequence and a viral leader sequence followed by unique restriction sites for cloning and a nopaline synthase terminator sequence. The complete expression cassette containing the promoter-gene fusions was cloned in the binary expression vector[29] pBINPLUS using the *Asc*I and the *Pac*I restriction sites.

Table 1: Designed oligos for building the construct (based on the sequence from AF020542, DBGET, GenomeNet, 2000; primers 1 to 3) and for testing the presence of the construct in putative transformants (primers 4 and 5)

primer 1 (All1HpaI) (5'-onwards oligo introducing H*pa*I site):
5' CCAACA**GTTAAC**TCTCAACCTTCACTAAAACCATCATCC **3'**

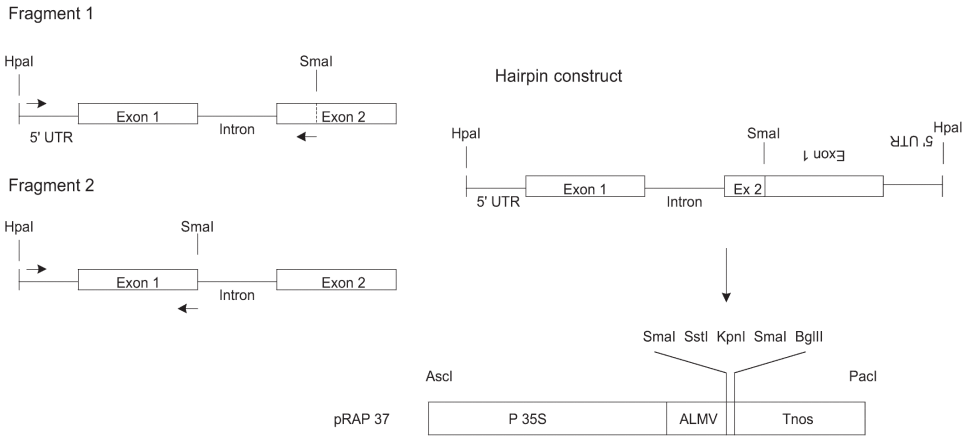
primer 2 (All2SmaI) (5'-onwards oligo for 3'-end introducing S*ma*I site within Exon 2)
5' CAAAG**CCCGGG**CGTATGAGTAGTTTGCTTCGTCAACC **3'**

primer 3 ((5'-onwards oligo for 3'-end introducing S*ma*I site at the end of Exon 1)
5' CTGACCTTCA**CCCGGG**GTGATCTTTTG **3'**

primer 4 (Apple 1)
5'-GCAAACTACTCATACGCCCGGGTGATCTTTTG-3'

primer 5 (Tnos-50)
5'-ATGATAATCATCGCAAGACCG-3'

Figure 1



Construct for gene silencing. The construct was built by linkage of Fragment 1 (obtained by PCR using primer 1 All1HpaI and primer 2 All2SmaI) and Fragment 2 (from primer 1 AllHpaI and primer 3 All3SmaI) at the SmaI-site and insertion in the expression cassette pRap 37. This cassette was cloned in the binary expression vector pBINPLUS and introduced in *Agrobacterium tumefaciens*, strain AgI-0, for further transformation of *in vitro* leaf explants of the apple cultivar Elstar.

Transformation

For transformation, the protocol was used according to Puite and Schaart was used[30]. In three independent transformation experiments, leaf explants from *in vitro* culture of the cultivar Elstar were placed for 30' in liquid MS20 medium at pH 5.2, containing a log-phase suspension of *Agrobacterium tumefaciens* strain Agl-0 carrying the pBINPLUS vector, and then transferred to fresh MS20 medium for four days (co-cultivation). Next, the explants were placed on shoot induction medium (SIM) containing kanamycin (150 mg/l) and cefotaxim (250 mg/l) to select for outgrowth of only the transformed plant cells and to kill *A. tumefaciens* cells. After callus formation, calli were isolated and subcultured individually. Newly appearing shoots were cut off from the callus and subcultured on shoot elongation medium (SEM). Elongated shoots were transferred to shoot propagation medium (SPM). In SEM and SPM, the kanamycin was omitted. The transformation experiments were carried out under the project GGO 98-123 (Plant Research International). Finally, putatively transformed shoots were tested by PCR for the presence of the construct according to Greene *et al.*[31] and adapted for miniprep isolation. Genomic DNA was isolated by grinding leaf material under liquid nitrogen, transferring the powder in urea extraction buffer, extracted with phenol-chloroform-isoamyl alcohol, and precipitated. To test for the presence of the construct in the isolated DNA, PCR was carried out using the 5'–end primer 4 (Apple1) and the 3'–end primer 5 (Tnos -50), resulting in a 330 bp fragment (see Table 1 for the primer sequences). For amplification, the DNA was denatured at 94^0 C for 1 min. and subjected to 35 cycles of PCR using SuperTaq polymerase (Sphaero Q). Each PCR cycle consisted of denaturation at 94 ºC for 30 sec., annealing at 58 ºC for 30 sec., and primer extension at 72 ºC for 1 min. After the last cycle, the samples were incubated at 72°C for 7 min for a final extension step. PCR products were loaded on gel. Shoots showing the presence of the construct were used

for further analysis in skin prick tests and immunoblotting. Three *in vitro* plants that lacked the construct, as well as the untransformed wild type (WT) plant were used as controls for normal Mal d 1 expression. In addition, an apple fruit from the cultivar Fiesta was used as a control in SPT.

Skin prick test (SPT)

Three adult patients with a history of birch pollinosis and apple allergy were recruited via the outpatient clinic of the University Medical Center Utrecht (UMCU). Apple allergy was confirmed in these patients by a positive double-blind placebo-controlled food challenge (DBPCFC) with apple, a positive skin test (wheal surface of > 7 mm^2) and/or a positive RAST (> 0.7 IU/ml) for birch pollen and apple. All patients had seasonal rhinitis durind the period of February - April, the tree pollen season in The Netherlands. SPTs were performed on the flexor surface of the patient's forearm using a standardized prick needle (ALK Lancet) according to Dreborg[32,33]. The transformed and control *in vitro* plantlets were applied using the prick-to-prick method. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent, normally applied for SPT-extracts, was used as a negative control (ALK-ABELLÓ, Nieuwegein, The Netherlands). SPT reactivity was measured after 15 minutes by copying the wheal reaction with transparent adhesive tape onto a record sheet for later comparison. The skin wheal areas were evaluated by computer scanning according to Poulsen *et al.*[34]. SPT results were expressed relative to the histamine control. SPTs were regarded positive when this ratio was ≥ 0.25, i.e. when the wheal induced by the plantlet was at least 25% of that of the positive control. The study was reviewed and approved by the local medical ethical committee. Informed consent was obtained from all subjects before enrolment in the study.

SDS-PAGE/Immunoblotting

Equal amounts of leaves from plantlets were homogenized directly in sample mix and heated at 100 °C for 10'. 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, California, USA). Western blotting was performed by semi-dry transfer to nitrocellulose on a Novablot electrophoretic transfer apparatus according to the manufacturer's instructions (Invitrogen). For detection of Mal d 1, either monoclonal antibody 5H8 directed to Bet v 1 and cross-reactive to Mal d 1 or a pool serum of birch pollen and apple allergic patients was used. After blocking with PBS/ 10 mM EDTA/0.3% BSA for a minimum of 10 minutes, blots were incubated overnight with 150 µl serum pool or 10 µl mAb 5H8[35] in 3 ml of PBS-AT. After washing with PBS/ 0.1% Tween-20, radiolabeled sheep antibodies against human IgE or goat antibodies against mouse IgG (Sanquin, Amsterdam, The Netherlands) were used for detection of bound IgE or mAb 5H8, respectively. Blots were exposed to X-ray film (Eastman Kodak Company, Rochester, NY, USA).

Statistical analysis

Statistical analysis were carried out on log-transformed SPT values. The log-transformation was chosen in order to obtain observations with an approximate constant variance; the variance

of the SPT values is approximately proportional to the mean. For the log-transformed SPT, a value of zero corresponds with the reaction to histamine. Values smaller than zero indicate a less severe reaction than the reaction to histamine. In all situations analysis of variance (ANOVA) was carried out using the computer package Genstat. All paired comparisons were made using a two-sided t-test.

Results

Plant transformation

From three independent transformation experiments including a total of 2,770 Elstar *in vitro* leaf explants and the suspension of *A. tumefaciens* strain Agl-0 carrying the gene-silencing construct, about 1,000 calli were formed after growth on shoot induction medium (SIM). Almost 340 of these calli produced shoot-like structures. These structures were initially vitrified in appearance; this disappeared only in some of the structures after subculturing. Their growth was very slow. Slow growth and vitrification are normal phenomena in the shoot regeneration process from apple calli. Fifty-seven shoot-like structures developed further and were transferred to shoot elongation medium (SEM). Of these, 17 produced very small plantlets with a normal phenotype that were subsequently transferred to shoot propagation medium (SPM) for further outgrowth. The best growing plantlets were subcultured to be used for analysis of the presence of the construct for gene-silencing, and for application in SPT and for further immunoblotting. These plantlets were A11, A12, B1, B2, B4, D2, D3, F1, G1, and the non-transformed WT. PCR analysis revealed that D2, D3 and F1 did not contain the construct. These lines were further used as positive controls for gene-silencing, in addition to the WT. The phenotype of the transformed plantlets was indistinguishable from the WT.

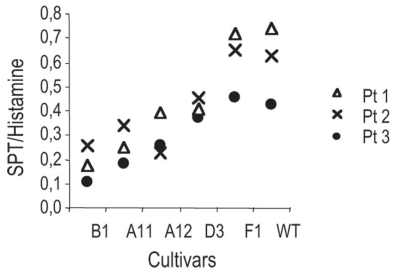
Assessment of hypo-allergenicity of transformants by SPT

Six plantlets with established presence of the gene-silencing construct and the controls (WT, D2, D3 and F1) were evaluated for their allergenicity by prick-to-prick SPT in three birch pollen and apple allergic patients. In addition to the *in vitro* plantlets controls, prick-to-prick testing using an apple fruit from the cultivar Fiesta was done as a further positive control. Figure 2 shows the consistently reduced allergenicity of the transformants A11, A12 and B1 in all three patients. In several cases the skin reaction was scored negative (<0.25). This was observed 2/3 times for B1 and 1/3 times for A11 and A12. The transformants (except B4) induced a significantly reduced SPT response compared to the control plantlets (Table 2). WT and F1 showed a degree of biological activity close to that observed for the Fiesta apple.

Table 2: Mean SPT/Histamine reactivity and the standard error of the mean of six transformants and five controls (D2, D3, F1, WT and Fiesta fruit)

Plant	Mean SPT/Histamine	SEM
B1	0,18	0,05
A11	0,26	0,05
G1	0,28	0,10
A12	0,29	0,05
B2	0,31	0,11
B4	0,38	0,11
D3	0,41	0,03
D2	0,46	0,15
WT	0,60	0,09
F1	0,61	0,08
Fiesta	0,86	0,07

Figure 2

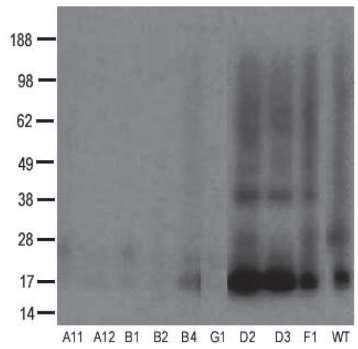


SPT reactivity of three transformants and three control plantlets in three patients. Reactivity was expressed relative to the histamine control.

Immunoblot analysis: significant reduction of Mal d 1 expression

To further confirm that expression of Mal d 1 was indeed significantly reduced, extracts of all plantlets were evaluated by immunoblot analysis after separation by SDS-PAGE (Figure 3). All transformants showed little (B4) or no reactivity on immunoblot with mAb 5H8. In contrast, this mAb detected a clear band at the expected M_r of approximately 17 kDa for WT, D2, D3 and F1. A serum pool with specific IgE against Mal d 1 showed weak but significant reactivity with the same band. This was not observed in the transformants, confirming the hypo-allergenicity of these plantlets.

Figure 3



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Cross-reactivity of the monoclonal antibody 5H8 (directed to Bet v 1) to Mal d 1 in six *in vitro* grown transformants and four control plants. Mal d 1 was separated by SDS-PAGE. For detection, radiolabeled goat antibody against mouse IgG was used.

Discussion

In this study we have successfully demonstrated the feasibility of reduction of Mal d 1 expression by RNA interference (RNAi). The efficiency of transformation of apple plants has been reported to be low[30]. Indeed in our study only a few dozen plants were obtained from almost three thousand original explants. Of these plants, only a limited number (5) showed the desired effect of strongly reduced expression of Mal d 1. The gene-silencing construct was designed on the basis of sequence information of Mal d 1 from Gala, but was capable of silencing Mal d 1 in Elstar. Perhaps the efficacy of gene-silencing could be improved if the design of the construct were based on sequence information from the target cultivar. At the time the present study was carried out, the cultivar Gala was not available for transformation. Despite this mismatch, silencing was successful, suggesting that the requirements for sequence homology are not extremely stringent. This notion is also supported by the fact that eighteen loci for Mal d 1 genes have recently been identified on the apple genome (Gao *et al.*, personal communication). Apparently, the degree of homology with the introduced gene-silencing construct was sufficient to silence a variety of endogenous Mal d 1 mRNAs. The potential of RNAi to silence complete gene families has also been demonstrated in wheat, where a conserved alpha gliadin sequence, cloned into an RNAi construct, was able to silence the entire alpha-gliadin gene family in a hexaploid wheat variety[36].

The effect of gene-silencing was monitored by both *in vivo* skin prick testing and immunoblotting using leaves of apple plantlets growing *in vitro*. The fact that Mal d 1 has been shown to be expressed in leaves as well as in fruits allowed us to screen for effective gene-silencing at a very early stage of plant development. If screening were dependent on full-grown fruit-bearing trees (which take many years to grow from *in vitro* plantlets or from seed), the production of hypoallergenic apples by gene-silencing would most likely not be feasible.

At this stage it was not possible to reliably quantify the level of reduction. The prick-to-prick method does not allow titration of skin testing. Immunoblotting suggested that the

reduction of expression was at least 10-fold, but this technique is only semi-quantitative. We performed a competitive RIA using mAb 5H8[35] and radiolabeled purified Mal d 1 in an attempt to quantify Mal d 1. Results of this experiment were inconclusive. Compared to the unsuccessful transformants F1 and D3, hypoallergenic plantlets A11 and A12 showed at least a 100-fold decrease in Mal d 1 levels. For B1 this was at least 10-fold. Surprisingly, the WT leaf extract had 100 times lower Mal d 1 activity than F1 and D3. Most likely this discrepancy is related to the problems we encountered in efficiently and reproducibly extracting the plantlets. Quantification therefore has to be repeated when larger amounts of leaf material become available.

Despite the lack of reliable quantification, our observations have indicated that further research into RNAi as a means of producing hypoallergenic apple cultivars certainly is promising. Of course, new studies will have to be extended to the phase of fruit bearing trees. At least at the *in vitro* plantlet stage, no phenotypical abnormalities were observed. This suggests that there is a good chance that healthy fruit-bearing plants can be grown. Since the Mal d 1 gene family (Gao *et al.*, personal communication) codes for proteins that are related to plant defence (pathogenesis-related PR proteins)[37], the effects of the current level of Mal d 1 silencing on the strength of defence against microbial pathogens and other stress-causing stimuli still needs further study.

Recently, several new plantlets have been developed from the calli. These plantlets, together with the most promising plantlets from the present study, will now be grown to sizes that will allow confirmation and better quantification of the observations reported here.

Acknowledgements

Thanks are due to drs Gerard Rouwendal and Jan Schaart for their advice in building the RNAi construct and apple transformation, respectively. Dr. Hans Jansen was very helpful in regard to the statistical analysis of the SPT results. Further, we are grateful to the patients for their participation in this study. The study was supported by a grant from the EC to the EU-SAFE project (QLK1-CT-2000-01394) and a grant from the Dutch Ministry of Agriculture, Nature Management and Food Safety within the frameworkof the DWK programme 408 on 'Plant compounds and health'.

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7

Attitudes towards low allergen food in food allergic consumers

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Submitted

Abstract

Background: The aim of this research was to look at food allergic consumers' preferences concerning the development of low allergen food.
Methods: A questionnaire was designed to measure attitudes towards low allergen food in food allergic consumers. Data were collected from twenty food allergic consumers in each of Austria, Spain and the Netherlands between April and May 2002 using interviewer-assisted questionnaire methodology.
Results: The results suggested that food allergic consumers are interested in having low allergen food available, with 70-95% of the sample wanting low allergy food to be produced. 89% of the participants in this study identified a number of benefits to themselves, such as being able to eat the food to which they were allergic again, and being able to eat all food with no worries, no symptoms and no need to check labels. Fewer disadvantages were mentioned, with 53% of the sample identifying no disadvantages. A number of factors that would encourage them to buy, or discourage them from buying low allergen food were also identified with price, quality (particularly taste) and safety being important factors. Whilst acceptance of low allergen food produced using genetic modification was reasonably high (55-85%), in general participants would prefer this food to be produced through conventional means.
Conclusions: The majority of food allergic consumers are interested in having low allergen food available. But concerns, particularly about price, quality and safety, would need to be addressed. Though genetic modification is acceptable, there is a preference for low allergen food to be produced through conventional means.

Introduction

It is widely acknowledged that in industrialised countries food allergy affects around 1-2% of the total population and up to 8% of young children, equating to 8 million food allergic individuals in the European Union[1]. For most food allergic individuals the impact of their condition is on quality of life, with a small, but significant, number of people suffering more severe reactions (which can include anaphylaxis and death).

Whilst various methods are being examined for their potential therapeutic value in treating food allergy[2-6], currently the only way of treating a food allergy is through an elimination diet, that is avoiding the offending food[7]. "The burden of living with food allergies starts as soon as the patient leaves the diagnosing physician's office. In the absence of a proactive treatment of food allergies, the instructions are as follows: "Avoid completely all foods containing the offending allergen." "[8]. [As implied by this quote, the effect of suffering a food allergy on quality of life is no small thing. It has the potential to impact on every aspect of the food allergic individual's life, and on their wider family and social circle [see [9] for further discussion of this issue].

Research is being conducted to look at various ways to produce hypoallergenic (low allergen) or non-allergenic foods[10,11]. Hypoallergenic milk (in the form of partially and extensively hydrolysed formula) is already available for infant food allergies, or a high risk for development of food allergies. One method that could be used to produce low allergen foods is genetic modification[12-14]. It has been argued that the use of genetic modification to eliminate, or reduce, allergenicity of existing food would be seen as a considerable benefit by consumers[15]. However, as yet, there is little evidence for this assertion.

Irrespective of the method used to produce the low allergen or non-allergenic food, there is an as yet unmet need to investigate the attitudes of food allergic consumers to having this food present. We need to know, for example, if food allergic consumers see any benefit to having such food available, whether or not they would buy it, and what would encourage and discourage them from purchase. This kind of information is important for the food industry to know when developing new products to ensure that the industry is able to sell its products, and critically that the food allergic individual gets the type of products of most use. Thus, the aim of this small, exploratory study is to begin to look at whether of not food allergic consumers are interested in having low allergen food available, and the factors that influence their decision.

Research aim

The primary aim of this research was to investigate attitudes towards low allergen (hypoallergenic) food in food allergic consumers in Austria, Spain and the Netherlands. A secondary aim was to examine preference for production method.

Material and Method

Materials

A questionnaire was designed to measure attitudes towards low allergen food in food allergic consumers. A mix of closed and open-ended questions was used; the use of open-ended questions allowed the participants to express their own views, in their own words (see Appendix). The questionnaire was in five sections and included items designed to measure: (A) demographic characteristics; (B) background to the patient's food allergy and effects on lifestyle; (C) perceived benefits and disadvantages of low allergen food; (D) attitudes to methods of producing low allergen food (genetic modification versus conventional plant breeding); and (E) preferences for receiving information about low allergen food. Before answering any questions in Section B, participants were given a brief explanation of what the researchers meant by low allergen foods: "foods which are low in allergens, this means that they contain less of the substance that causes an allergic immune response in people allergic to these foods." In Section C participants were asked if they had heard of genetic modification. Following this all the participants were given a simple explanation (see Appendix) of what genetic modification is, and concrete examples of how it has been used in agriculture for food production purposes. This was to ensure that all participants were answering the remaining questions with the same information about genetic modification.

Design and conditions

A between subject design was used where food allergic consumers were interviewed in one of three different countries: Austria, Spain or the Netherlands. An interviewer-assisted questionnaire design was used, where clinicians asked the participants the questions and recorded their responses.

Procedure

The data were collected between April and May 2002. The study took place within the allergy department of the hospital. The patients were recruited by the interviewing clinician. No one approached to take part in the study refused. Some of the participants were already part of the European Commission funded SAFE project on apple allergy; others were attending the hospital for their routine allergological evaluation or for immunotherapy. The questionnaire took between 15 and 30 minutes to complete.

Participants

Sixty participants took part in the research, twenty in each of the three countries. The age of the participants ranged from 17 to 58 (mean 32.12±10.35), with 23 (38%) men and 37 (62%) women participating (demographic data for individual countries can be seen in Table 1).

Table 1: Demographic characteristics by country

Demographic characteristic		Austria	Spain	The Netherlands
Gender	Male	8 (40%)	7 (35%)	8 (40%)
	Female	12 (60%)	13 (65%)	12 (60%)
Age	Range	21-58	18-55	17-54
	Mean (SD)	34.25±10.59	28.05±8.43	34.1±10.34

All the patients were diagnosed for their food allergies using SPT (skin prick test) and case history (and measures of specific IgE in Austria and Spain). Some of the food allergies were confirmed by open food challenge (OC) or double-blind placebo controlled food challenge (DBPFC) in Spain and the Netherlands.

Results

Background to participants' food allergy

Ninety five percent of the sample were allergic to more than one food (range 1-24, mode = 2), with the most common being apple (Austria = 15 participants, Spain = 16, The Netherlands = 20)*. Fifty-three foods were mentioned in total; mostly fruit (24 different types of fruit), but also vegetables, fish, cereal crops, alcohol, spices and seeds. All of the participants had problems with whole food and raw food. Fewer had problems with the allergic food as an ingredient in processed food (Austria = 9 (45%); Spain = 13 (65%); The Netherlands = 13 (65%)), or cooked food (Austria = 4 (20%); Spain = 6 (30%); The Netherlands = 4(20%)).

The participants were asked about the history, severity and treatment of their food allergy. Over a fifth (23%) of the sample had had their food allergy diagnosed by a medical professional the same year that they suspected they had a food allergy. However, a third of the sample (33%) was not diagnosed for up to five years, and 43% were not diagnosed until after five years. Twenty-three of the sample had family members with food allergies (Austria = 9, Spain = 2, The Netherlands = 12). Most family members mentioned were first degree relatives (parents, siblings or children).

Almost all of the participants dealt with their food allergy by avoiding the food to which they are allergic (97%), some also avoided other food products containing this food (18%) and checked food labels (28%). However, nearly two thirds (65%) claimed that their food allergy had no effect on their lifestyle. It is likely that this was due to the types of food these participants were allergic too (for example, peanut allergy is generally seen to have a large impact on quality of life, but only 10 of the participants were allergic to peanuts). Where there was an impact on lifestyle, it related to not being able to eat the food and to eating out. There was generally no effect on their job, playing sport, or where they went on holiday.

More of the Austrian participants rated their allergic symptoms as mild than in the other two countries (Mild: Austria = 9 (45%), Spain = 4 (20%), the Netherlands = 4 (20%); Moderate:

Austria = 6 (30%), Spain = 6 (30%), the Netherlands = 8 (40%); Severe: Austria = 5 (25%), Spain = 10 (50%), the Netherlands = 8 (40%)). None of the Dutch sample had been hospitalised (or visited the emergency room of the hospital) for emergency treatment for their food allergy; 2 (10%) of the Austrian sample and 12 (60%) of the Spanish sample had been hospitalised (or visited the emergency room of the hospital). Generally, few of the sample took any medication for their food allergy.

Attitudes to low allergen food

Over eighty percent (83%) of the sample would like low allergen foods to be produced; this was slightly higher in Spain (95%) and the Netherlands (85%) than in Austria (70%)[1].

In assessing preferences for production method of low allergen food five steps were followed. Firstly, the participants were asked if they would buy a low allergen food produced using conventional plant breeding. Secondly, they were asked if they had heard of genetic modification. Then they were given a definition of genetic modification within the context of food to read. Next they were asked if they would buy a low allergen food produced using genetic modification. Finally, they were asked about their preference for low allergen food production. Overall, two thirds (77%) of the sample had heard of genetic modification; this was higher in Austria (85%) and the Netherlands (80%) than in Spain (60%).

Table 2: Desire for low allergen food by country

	Austria	Spain	The Netherlands	Total
Production method	**Number who would purchase**			
Using conventional plant breeding	19 (95%)	18 (90%)	20 (100%)	57 (95%)
Using genetic modification	11 (55%)	17 (85%)	16 (80%)	44 (77%)
Preferred method	**Number who preferred each method**			
No preference	4 (20%)	6 (30%)	6 (30%)	16 (27%)
Genetic modification	0	2 (10%	1 (5%)	3 (5%)
Conventional plant breeding	16 (80%)	11 (55%)	13 (65%)	40 (67%)
Neither	0	1 (5%)	0	1 (2%)

Almost all the participants said that, if available, they would buy low allergen food produced by conventional plant breeding, and three-quarters would buy low allergen food produced by genetic modification; this was much lower in Austria where only half the sample would buy low allergen food produced using genetic modification. However, across all three countries, when asked to express a preference two thirds of the participants (67%) would prefer low allergen food to be produced using conventional plant breeding (Table 2).

Benefits and disadvantages of low allergen food

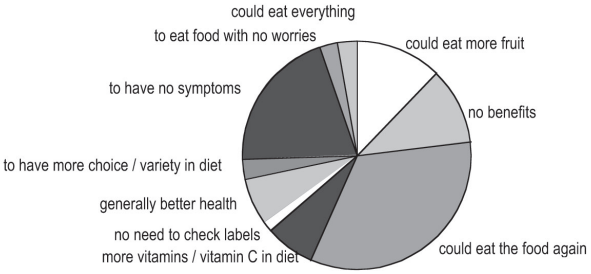
The participants were asked to identify the main benefits to themselves of being able to purchase low allergen foods, and the main disadvantages in two open ended items. They identified a number of benefits to having low allergen food available, including:

- Being able to eat the food to which they were allergic;
- Being able to eat with no worries, no symptoms and no need to check labels;
- Being able to eat everything (i.e. all food);
- Having more variety, more fruit and more vitamins (particularly vitamin C) in the diet;
- Having generally better health

Only 11% could identify no personal benefits to having low allergen food available (Figure 1).

There were some cross cultural differences, in that the Spanish and Austrian samples were more likely to identify being able to eat the food to which they were allergic again as a personal benefit than the Dutch. Additionally, more of the Austrian sample specifically mentioned being able to eat more fruit than in the other two countries. The Dutch were more likely to mention having no symptoms has a benefit than participants in the other two countries.

Figure 1. Benefits of having low allergen food available



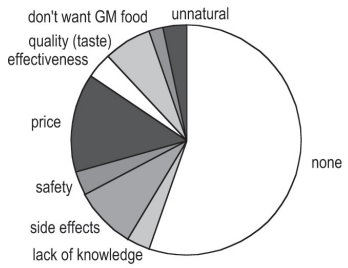
A number of disadvantages to having low allergen food available were also identified, including:

- They may cost more.
- They may cause unknown side effects, such as developing new allergies, developing new diseases.
- The quality, particularly the taste, may not be as good.
- General concerns about the safety of low allergen foods.
- Producing low allergen food is manipulating nature, and unnatural.
- There is a general lack of knowledge (e.g. about the risks & benefits, what is in the food).
- They may not actually work properly i.e. they would not be hypoallergenic.
- A desire not to have genetically modified food**.

However, over half of the sample (53%) could identify no disadvantages (Figure 2).

There were a few cross cultural differences in the type of disadvantages identified by the participants in the three countries. In all three countries around half the sample stated that there were no disadvantages to having low allergen food available. The various safety aspects (side effects, safety, not effective) were slightly less of a concern for the Spanish sample than participants in the other two countries. Price was an issue for two or three people in all three countries. Taste and quality were not important in Austria (mentioned by no participants). The possibility of the low allergen food being genetically modified, the food being unnatural, and a general lack of knowledge about low allergen food were only concerns in Austria.

Figure 2. Disadvantages to having low allergen food available



Factors that would encourage and discourage purchase

The allergic individuals were also asked what would stop them from buying low allergen food, and what would encourage them to buy low allergen food in a further two open ended items. It was found that price was an important issue; people wanted the low allergen food to be good value for money, and a high price would discourage them from buying. Quality, particularly taste, was also important. Critically, the participants wanted to be sure that the food was safe. Other less frequently mentioned factors that would stop people from buying low allergen food included: if the food was unnatural, if the food was produced using genetic modification[3], if the food was tested on animals, if people did not have enough information about the food, and if the expiration date was short. People would be encouraged to buy low allergen food if they were allergic to a staple food (e.g. bread, milk), or if they had severe symptoms. Most people would want to buy low allergen food simply because it was low allergen, and thus would provide the benefits mentioned previously, such as being able to eat the food to which they are allergic safely, with no symptoms and having more choice in their diet, being able to eat all food, giving them a better quality of life.

There were a few cross cultural differences in that price and taste were the biggest drivers in the Netherlands in terms of what would stop purchase. Price and safety were the biggest drivers in Spain, but more of the participants in this country claimed that nothing would stop them buying low allergen food than in the other two countries. Price and safety were also the biggest drivers in Austria, but naturalness and an aversion to genetically modified

foods were also issues. As well as being a benefit of low allergen food, the chance to have no symptoms was the biggest encouragement to purchase for the Dutch sample. The same was true for being able to eat the food again in Spain. The factors encouraging the Austrian sample were more varied with issues of quality, being good value for money and having more choice in the diet being important as well as being able to eat the food again and having no symptoms.

Information about low allergen food

Participants were asked how they would like to be informed and from whom they would like to receive information about both new scientific developments in growing low allergen foods, and the availability of low allergen foods in two open ended items.

Most allergic individuals wanted information about new scientific developments in growing low allergen foods from medical doctors (70%). Other medical sources (e.g. pharmacy, hospital/clinic) were also mentioned (13%), as were other experts (e.g. scientists) (15%). Supermarkets (5%), friends (3%) and patient groups (3%) were less frequently named. The most popular means of getting this information was via the media (30%). Also popular were the internet (15%) and leaflets (10%). Direct mailing (7%), talking to people (7%), lectures (2%) and email (2%) were less frequently mentioned.

When asked who should provide information about the availability of low allergen foods, medical doctors (40%) and the food industry (food companies, organic shops and particularly supermarkets) (13%) were popular. Other medical sources (10%), other experts (15%), friends (5%) and patient groups (3%) were mentioned again. The preferred means of getting information about availability was through leaflets and the media (30% each). Also mentioned was the internet (17%), direct mailing (8%), talking to people (7%), email (3%), and newsletters (3%).

Labelling of low allergen food

When asked how they would like low allergen foods to be displayed or labelled in the shops 87% of the sample wanted low allergen food to be identified by a label (in the Netherlands, no further details were provided), and there was specific reference to the need for *clear* labels (particularly in Spain). In Spain and Austria some of the sample wanted a logo on the label, others wanted everyone to use the same way of labelling, others wanted the process used to produce the low allergen food put on the label. In addition, some of these participants wanted low allergen food to be displayed in a separate part of the shop. The displaying and labelling of low allergen food was compared to organic produce by some of these participants.

When asked if they would trust that manufacturers were telling the truth if they labelled food as 'low allergen' most of the participants in Spain (75%) and the Netherlands (80%) said that they would trust manufacturers (Figure 4). However 50% of the Austrian sample (only 30% responded that they would trust manufacturers) responded with stipulations. These participants claimed that they would accept low allergen labelling on food only if it was

checked (and approved) by someone else, specifically an independent, expert, national or international organisation. They also wanted the food to be EU (European Union) or WHO (World Health Organization) approved, regular checks of the validity of low allergy claims, well controlled testing of the food. Furthermore, they wanted the information to be available from the media too.

Discussion

The primary aim of this study was to investigate attitudes towards low allergen food in food allergic consumers in Austria, Spain and the Netherlands. The findings indicate that, generally, attitudes towards having low allergen food available were positive; suggesting that the development of such products would be welcome by this consumer group.

This was a small sample, exploratory study, and as such these results can not be taken as generalisable to all food allergic consumers, even within these three countries. This said, the allergic individuals' free responses provide useful information about the kinds of issues that are important to them within the context of low allergen food. Such information is likely to be useful for the potential developers of low allergen food products to ensure that the needs of the allergic consumers are met, benefiting these consumers and the food industry. The data collected could usefully be validated with a large sample of respondents.

The results of the study point towards a number of consumer identified benefits and disadvantages of having low allergen food available. The key benefits identified included being able to eat the food to which they were allergic again and having no symptoms. For the majority of participants the simple fact that the food was 'low allergen' and would provide these, and other, benefits was enough to encourage them to purchase low allergen food. Many of the participants could see no disadvantages to having low allergen food available. The main disadvantages identified related to the possibility of a high price, of poor taste and quality and of the product not being safe in some way (e.g. causing side effects, and not being effective). The factors that would discourage people from buying low allergen food strongly mirrored these potential disadvantages. Thus, these concerns would have to be addressed to enable food allergic consumers to feel comfortable buying low allergen food products. The issues relating to clear, consistent and informative labelling of low allergen food, and their placement and advertisement would also need to be dealt with. There is a suggestion that in Austria simple labelling of low allergen food is not enough, and that independent verification of low allergen claims and information about this is needed. It may be that such information would be welcome in other countries too. The preferences for information about aspects of low allergen food from medical doctors as well as the various components of the food industry suggest that low allergen food may be seen as a 'health' product as well as a food product. As such, a close interaction between these two information sources would be beneficial.

A secondary aim of the study was to investigate preference for production method for low allergen food. It was found that almost all participants would be happy to purchase low allergen food produced using conventional means. In Spain and the Netherlands a

considerable number would also purchase low allergen food produced using genetic modification. However, in Austria nearly half would not. Given a choice between these methods, two thirds of the sample would prefer conventional means to be used to produce low allergen food. Again, this was higher in Austria, suggesting the possibility of a more negative attitude towards genetically modified food in this country. This preference for conventional methods to be used to produce low allergen food is unsurprising. Previous research has indicated that whilst people do see the potential for benefit from genetically modified food, attitudes are generally negative[16]. A survey conducted with food allergic consumers in the UK found that a third claimed they would eat a non-allergenic food produced using genetic modification, a third would not, and a third were not sure. But over 80% were concerned that genetic modification may accidentally lead to new food allergies[17].

It must be remembered that, firstly, this is a small sample and, secondly, that there is a suggestion that cross-cultural differences exist in attitudes towards, and acceptance of, genetically modified low allergen food. However, with this in mind, even with the preference for conventional production methods, the fact that over three quarters of the entire sample claimed that they would purchase low allergen food produced using genetic modification hints that genetically modified food providing a specific benefit to a particular user group may be acceptable, or at least more acceptable than genetically modified food providing non-specific, or non-consumer benefits (e.g. benefits to the biotechnology industry). This issue would benefit from further research, with large samples to allow for cross-cultural comparisons.

Conclusion

The results of this study suggest that food allergic consumers are interested in having low allergen food available. Concerns about price, quality (particularly taste) and safety would need to be addressed. Whilst acceptance of low allergen food produced using genetic modification was reasonably high, in general participants would prefer this food to be produced through conventional means.

Acknowledgements

The research reported here was financially supported by the EC FP5 “Quality of Life and Management of Living Resources” program within the project “Plant food allergies: field to table strategies for reducing their incidence in Europe” (acronym SAFE; QLK1-CT-2000-01394). However, the views expressed in the paper are those of the authors.

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Appendix

INTERVIEWER READ THE FOLLOWING STATEMENT TO THE PATIENT
"Thank you for agreeing to take part in this research. I am going to ask you some questions about your food allergy, how it effects the way you live and what you think about low allergen food. I am interested in your opinions, there are no right or wrong answers."

A. Demographic questions

1) Gender *(male/female)*
2) Age (in years)
3) Highest educational qualification obtained

B. Preliminary questions

1) What food/s are you allergic too? *(Open ended)*
2) Are you receiving medical care from a medical professional for your food allergy? *(yes/no)*
3) Would you say that, overall, your allergic symptoms are minor, moderate or severe? *(minor/moderate/severe)*
4) Have you had ever been admitted to hospital for your food allergy? *(yes/no)*
5) Do you take any medication because of your food allergy? *(yes/no)*
6) When did you first know or suspect that you had a food allergy? *(year)*
7) When was your food allergy first diagnosed by a medical professional? *(year)*
8) Do you have any family members with food allergies? *(yes/no)*
9) Who? *(Open ended)*
10) Does your food allergy mean that whole food, for example an apple, is a problem? *(yes/no)*
11) Does your food allergy mean that processed food - where the food is used as an ingredient, for example apples in apple pie or a cereal bar, is a problem? *(yes/no)*
12) Does your food allergy mean that cooked food is a problem? *(yes/no)*
13) Does your food allergy mean that raw food is a problem? *(yes/no)*
14) How do you deal with your food allergy? *(Open ended)*
15) How does your food allergy affect your lifestyle, in other words does affect the way you live? *(Open ended)*

C. Benefits and disadvantages

INTERVIEWER READ THE FOLLOWING STATEMENT TO THE PATIENT
"In the future it may be possible to produce foods which are low in allergens, this means that they contain less of the substance that causes an allergic immune response in people allergic to these foods"

1) Would you like these low allergen foods to be developed? *(yes/no)*
2) What would be the main benefits to you of being able to purchase low allergen foods? *(Open ended)*
3) What would be the main disadvantages to you of being able to purchase low allergen foods? *(Open ended)*
4) What would stop you buying low allergen foods? *(Open ended)*
5) What encourage you to buy low allergen foods? *(Open ended)*

D. Methods of producing low allergen foods

1) If available, would you purchase low allergen food produced by conventional plant breeding? *(yes/no), If no,* Why?
2) Have you heard of genetic modification? *(yes/no)*

INTERVIEWER GIVE DEFINITION OF GENETIC MODIFICATION TO READ
"Genes are nature's instruction book for building living things. Our genes account for many of our physical characteristics. Slight variations in a gene cause variations in the character it controls e.g. one form of the gene for eye colour gives blue eyes, while a slightly different form of the same gene results in brown eyes. Genes are made up of DNA and are found in the cells of all living things.

Genetic modification (sometimes referred to as 'genetic engineering') involves two main steps:
1) identifying and separating the gene that codes for a particular characteristic, and
2) putting that gene into another living thing.

Unlike conventional breeding, genetic modification can move single genes. It can be used to transfer genetic information from one species to another, e.g. from bacteria to a plant.

There are a number of examples of using genetic modification in agriculture. Crops have been modified to improve weed control (herbicide tolerant crops), to make plants resistant to specific insect pests, to increase the nutritional quality of the crop (rice containing vitamin C) and to improve keeping quality (reducing the softening of ripe tomatoes).

Some genetically modified foods are whole and unprocessed, such as tomatoes; others are crop plants processed to produce food ingredients or animal feed, for example soya and maize."

3) If available, would you purchase low allergen food produced by genetic modification? *(yes/no) If no,* Why?
4) Would you prefer low allergen food to be produced by conventional plant breeding or genetic modification, and why? *(Open ended)*

E. Information

1) How would you like to be informed about new scientific developments in growing low allergen foods and from whom would you like to receive this information? *(Open ended)*
2) How would you like to be informed about the availability of low allergen foods and from whom would you like to receive this information? *(Open ended)*
3) How would you like low allergen foods in the shops to be displayed or labelled? *(Open ended)*
4) Would you trust that manufacturers are telling the truth if they labelled food as 'low allergen'? *(yes/no) If no,* Why?

Notes

[1] It was not possible to perform to use the chi-square test to perform statistical analysis due to the small number of participants in each group.

8

Efficacy of birch pollen immunotherapy on cross reactive food allergy confirmed by skin tests and food challenges

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Clin Exp Allergy. 2004 May;34(5):761-9

Abstract

Background: The effect of birch pollen immunotherapy (IT) on cross reactive food allergies is controversial.

Objective: The aim of this study was to investigate the effect of birch-pollen IT on apple allergy and to evaluate recombinant allergens and double-blind placebo-controlled food challenges (DBPCFC) as monitoring tools.

Methods: Twenty-five adult birch-pollen and apple allergic patients were randomly divided into two groups, either receiving birch-pollen IT or symptomatic drugs only. IgE and IgG4 antibodies against birch pollen, apple, natural Bet v 1 and Mal d 1 were measured. In addition, SPT were performed using recombinant Bet v 1 and Mal d 1. Clinical outcome was evaluated by DBPCFC. $CD4^+CD25^+$ regulatory T cells (Tregs) were isolated from peripheral blood and tested in functional assays.

Results: Birch-pollen IT resulted in a significant decrease of SPT reactivity for rBet v 1 (30-fold) and rMal d 1 (10-fold) already after three months. IgG_4 antibodies were potently induced against Bet v 1, displaying cross reactivity to Mal d 1. VAS scores decreased > 10-fold in 9/13 patients of the IT group, with 3 patients converting to negative. In the control group no decrease was observed. Birch-pollen IT did not lead to detectable changes in the number or function of the $CD4^+CD25^+$ Tregs.

Conclusions: This trial supports claims that birch pollen IT also decreases allergy to foods containing Bet v 1-homologous allergens. Recombinant allergens and DBPCFC have proven to be useful tools for monitoring the effect of birch pollen IT on linked food allergies.

Introduction

Up to 70% of patients with birch pollen allergy develop an IgE-mediated food allergy to fresh fruits of the Rosaceae family, nuts or vegetables[1,2]. This food allergy is characterized by immediate itching in mouth and throat, sometimes associated with mild to moderate angioedema, and is also known as the oral allergy syndrome (OAS) [3,4]. The combination of pollen and fruit allergy is explained by cross-reactive IgE antibodies that recognize homologous allergens in birch pollen and fruits [5,7]. The primary sensitizer is the major birch pollen allergen, Bet v 1. Its fruit homologues are generally believed to lack the capacity to sensitize directly, because they are labile (sensitive to processing) and pepsin sensitive, i.e. easily digested in the stomach. Consequently, the spectrum of IgE specificities is broadest against the pollen allergen. Selective recognition of epitopes on fruit or vegetable homologues of Bet v 1 is extremely rare [8].

Immunotherapy (IT) for the treatment of birch pollen allergy has been a well accepted and successful therapy for many years. IT for the treatment of inhalant allergies was described in 1900 by Curtis *et al*.[9] and is now widely used as an effective treatment of pollen, animal, mite and also insect venom allergy [10]. The mechanism of allergen-specific immunotherapy is slowly being unraveled. It has been described that IT leads to a shift in specific IgE and IgG_4 concentrations; IgE concentrations tend to rise initially after IT and subsequently decrease over time, whereas IgG_4 concentrations increase during IT. Since IL-10 stimulates the production of IgG_4, IL-10 producing T cells may be involved in the induction of tolerance by IT [11]. IL-10 can be produced by different T cell subsets, such as type 1 regulatory T cells (Tr1), Th3 cells or $CD4^+CD25^+$ regulatory T cells (Tregs) [12,13]. Recent publications have speculated on the possibility that $CD4^+CD25^+$ Tregs are involved in the process of tolerance induction, such as following successful IT treatment [14,15].

In view of the cross reactivity between birch pollen and fruits and/or nuts, IT with birch pollen allergens might be expected to improve associated food allergies. It is a long-standing debate whether IT for inhalant allergies indeed has beneficial effects on accompanying food allergies [16-19]. Some studies reported improvement[16,17,19], while an other study found no beneficial effect of tree pollen IT on apple allergy [18] In a case report of one patient, Kelso et al. showed improvement of apple allergy after tree mix IT by open challenge[17]. In another study 56% of patients treated with tree mix IT reported improvement as evaluated by questionnaire[19]. Asero reported clinical improvement in 84% of the patients as judged by open apple challenge [16]. A study by Möller could not demonstrate a beneficial effect of birch pollen IT on apple allergy in children that were evaluated by questionnaire.[18] Considering these conflicting results there is a need for well-controlled studies.

The aim of this study was to investigate whether support can be found for cross-desensitization for apple during birch pollen IT. To focus on the structures that are at the basis of birch apple cross reactivity, humoral (IgE and IgG_4) and cellular (Treg) immune-reactivity was monitored both in vitro and by skin prick test using purified natural and recombinant Bet v 1 and Mal d 1. Previous studies had never used the gold standard for food allergy diagnosis, the double blind placebo-controlled food challenge (DBPCFC), to determine the clinical outcome of treatment. In our study the DBPCFC was for the first time applied to monitor the effect of birch pollen IT on cross reactive apple allergy.

Methods

Patients

Twenty-five adult patients (9 male and 16 female, mean age 35.6 years) with a history of birch pollen and apple allergy were recruited from the outpatient department of Allergology of the University Medical Center Utrecht in the period between August and October 2001. This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All patients gave written informed consent before enrolment in the study. Inclusion criteria were a positive skin test (≥7 mm^2) to birch pollen extract (ALK-ABELLÓ, Nieuwegein, The Netherlands) and fresh apple (Golden Delicious) and specific serum IgE concentrations of ≥ 0.7 kU/L to birch pollen and apple (CAP-FEIA, Pharmacia & Upjohn Woerden, The Netherlands). We randomized 25 patients into two groups: one group to receive birch pollen IT (n=13) and the control group to receive only symptomatic treatment (n=12). Both patient groups were similar with respect to sex, mean age, birch pollen SPT reactivity, IgE concentrations for natural Bet v 1 (nBet v 1) and natural Mal d 1 (nMal d 1) and the severity of symptoms after eating apple (Table 1).

Two patients of the control group (Table 1, patient 24 and 25) were excluded because they were not willing to participate in the study without receiving IT, so in the control group 10 patients were left. Patient characteristics of these two persons did not differ from the other patients in the control group.

DBPCFC

Clinical outcome was investigated by DBPCFC before start of IT in November 2001 (t=0) and after 1 year (t=12 months). On the day of the challenge only a light breakfast was allowed at least 2 hours before the first test meal. The challenges were performed in a clinical research setting equipped for resuscitation and monitoring of vital signs. During the challenge the patient was permanently accompanied by an investigator or a nurse. 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. Oral allergy symptoms were scored using visual analogue scales (VAS) ranged: 0-100 mm. An interval of 15 minutes was allowed between each test meal when no reaction occurred, or longer if (subjective) symptoms were still present. A challenge was regarded positive when subjective symptoms were reported for at least three times after active doses or when objective symptoms were observed[20]. Patients were kept for observation for at least one hour after resolution of symptoms. The challenge meals were prepared within five minutes before administration and contained: 4, 10, 40 or 120 grams of fresh 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 due to processing steps and were added for optimal blinding of taste. All patients were nose clipped while eating the test meals. The increasing apple doses (n=4) were randomly interspersed with placebos (n=4).

Table 1. Patient characteristics.

Pt no	Group	Sex	Age (yr)	Pollen sens.	Apple related symptoms (CH)	SPT Apple (mm^2)	SPT Birch (mm^2)	RAST Apple (kU/ml)	RAST Birch (kU/ml)
1	IT	F	18	B G	OAS RC CU	52	52	5.8	12.3
2	IT	F	28	B G	OAS	42	27	2.9	5.8
3	IT	F	30	B	OAS RC CU	11	31	4.8	11.4
4	IT	F	30	B	OAS	30	51	1.3	3.4
5	IT	F	31	B G	OAS RC	15	53	0.7	1.9
6	IT	M	46	B G	OAS	49	29	4.8	11.7
7	IT	F	44	*B*	OAS RC	22	47	8.2	23.8
8	IT	F	35	B G	OAS RC	48	67	6.8	14.1
9	IT	F	33	B G	OAS RC	61	64	0.8	0.7
10	IT	M	54	B	OAS RC	28	46	1.6	5.4
11	IT	M	26	B G	OAS	24	37	0.8	8.3
12	IT	F	36	B G	OAS RC	21	27	0.7	1.4
13	IT	M	45	B	OAS RC CU	55	15	2.2	2.9
14	CON	M	50	B G	OAS	15	38	2.7	5.8
15	CON	F	22	B G	OAS	17	35	7.0	13.1
16	CON	F	47	B G	OAS RC	43	26	7.2	27.3
17	CON	F	44	B G	OAS RC	29	34	9.9	17.8
18	CON	M	28	B G	OAS RC	49	23	3.7	11.6
19	CON	F	22	B G	OAS RC CU	42	37	5.3	26.1
20	CON	F	48	B G	OAS RC	20	36	1.8	14.5
21	CON	M	31	B	OAS RC	64	29	18.8	32.3
22	CON	M	35	B G	OAS	25	12	0.7	5.8
23	CON	F	35	B	OAS RC CU	25	32	2.1	3.4
24*	CON	M	37	B	OAS	30	45	1.1	3.4
25*	CON	F	33	B G	OAS	53	23	5.7	4.9

IT= immunotherapy group, CON=control group,
B=birch pollen, G=grass pollen,
CH= case history, OAS= oral allergy syndrome, RC= rhinoconjunctivitis, CU=Contact Urticaria
* patient was excluded after randomization

IT protocol

The patients of the IT group received a standardized aluminium hydroxide adsorbed birch pollen extract, Alutard SQ (ALK-ABELLÓ). All patients were given IT according to a modified cluster schedule, between November 2001 and January 2002, followed by a monthly maintenance injection of 100.000 standard quality units (SQ-U) for one year with a dose reduction of 50% during the pollen season. All patients were pretreated with an

antihistamine at least one hour before injection, since this reduces side-effects and has been reported possibly enhance efficacy[21].

Allergens

Natural Mal d 1 and Bet v 1were purified by monoclonal antibody affinity purification[22] Recombinant Mal d 1 and Bet v 1 were produced in *E. coli* and purified as described elsewhere[23].

SPT reactivity

Patients were evaluated at 3-monthly intervals during one year by SPT. SPT was performed on the flexor aspect of the forearm with a standardized prick needle[24] (ALK-Lancet). The reaction was measured after 15 minutes by copying the wheal reaction with transparent adhesive tape on to a record sheet. The skin wheal areas were determined by computer scanning[25]. SPTs were regarded positive when the area was at least 7 mm^2. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT-extracts was used as a negative control (ALK-ABELLÓ). We used the recombinant allergens and commercial extracts in serial dilutions; birch pollen extract (0.1-10.000 Bioequivalent allergy units (BAU/mL; ALK-ABELLÓ) and the recombinant allergens, rBet v 1 (0.0002- 20 ìg/ml) and rMal d 1 (0.02-100 ìg/ml) (Biomay, Vienna, Austria). Reactivity to apple was tested by prick to prick method using fresh apple (Golden Delicious).

IgE and IgG_4 RAST

Specific IgE-titres against nBet v 1, nMal d 1, apple and birch pollen extracts were determined every three months. RAST was performed as described previously [22,26]. In brief, 100 µg purified protein or 4 mg extract (dry weight) was coupled to 100 mg CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). Per test, 50 µl serum was added to 0.5 mg purified protein or pollen extract sepharose or 1.5 mg food extract sepharose in a final volume of 300 µl PBS/0.3% (w/v) BSA/0.1% (v/v) Tween 20 (PBS-AT). Immuno-detection was performed with ^{125}I-sheep anti-human IgE (Sanquin, Amsterdam, The Netherlands). To calculate RAST results in international units (IU) per mililiter an in-house standard of mouse/human chimeric IgE antibodies against Der p 2 was used. These antibodies were calibrated against the WHO international reference for IgE and tested in different dilutions for binding to Sepharose-coupled rDer p 2 to generate a standard curve. Patients were designated as sensitized if specific IgE concentrations were ≥ 0.7 IU/ml. IgG_4 antibodies against birch pollen and apple were measured using the same RAST protocol adapted for detection of IgG_4. To this end, 1.25 µl of serum was used instead of 50 µl. Detection was performed with radiolabeled monoclonal antibody against human IgG_4 (Sanquin).

Allergen binding RIA

IgG_4 antibodies against nBet v 1 and nMal d 1 were measured using an antigen-binding RIA with radiolabeled purified allergens[27]. Serum (1.25 µl/test) was incubated overnight with 0.5 mg anti-γ_4-Sepharose and radiolabeled allergen (~ 10.000 cpm) in a final volume of 350 µl

PBS-AT. Radioactivity, bound to the solid phase was measured on a gamma counter. Results were expressed in arbitrary units using a standard curve of a rabbit polyclonal antiserum against Bet v 1.

Isolation of PBMC and purification of CD4+ T cell subsets

PBMC were isolated from heparinized venous blood from the IT and control group one year after IT (t=12) and age-matched healthy controls (without atopic history) using Ficoll-Isopaque centrifugation (Amersham Biosciences, Uppsala, Sweden). Three subsets, namely $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ regulatory T-cells (Tregs) were isolated from PBMC using magnetic activated cell sorting (MACS). $CD4^+$ T cells were negatively selected with a CD4 T cell isolation kit obtained from Miltenyi (Bergisch Gladbach, Germany). To separate the $CD4^+CD25^-$ from the $CD4^+CD25^+$ T cells, the $CD4^+$ T cells were incubated with anti-CD25 microbeads (Miltenyi) and separated using a positive selection column. The various T cell subsets were tested in proliferation and suppression assays, as described previously[28,29]. Briefly, for proliferation assays, the proliferative response of $CD4^+$ T-cells depleted of the $CD25^+$ subset ($CD4^+$ $CD25^-$ T-cells) and $CD4^+CD25^+$ Tregs ($1x10^5$ T cells/well) was compared. Irradiated autologous PBMCs, depleted of $CD4^+$ T cells, were used as antigen presenting cells (APC). For suppression assays, $CD4^+CD25^-$ T-cells were stimulated in the absence (ratio 1:0, total amount of T cells/well $1x10^5$) or presence (ratio 1:1, total amount of T cells/well $2x10^5$) of autologous $CD4^+CD25^+$ Tregs. The following stimuli were used: 0.04 μg/ml anti-CD3 mAb (Pelicluster CD3 culture supernatant, Sanquin), birch pollen extract (50 μg/ml), rBet v 1 (5 μg/ml), rMal d 1 (5 μg/ml) and 50 μg/ml purified cow's milk antigen (consisting of casein, α-lactalbumin and β-lactoglobulin) (NIZO, Ede, the Netherlands). Cells were cultured for 3 days with anti-CD3 mAb or 5 days with specific antigen. During the last 18 hours of the culture period, [^{3}H]-TdR was added at 1 μCi per well (Amersham, Aylesbury, UK). Proliferative responses of the $CD4^+$ $CD25^+$ T cells alone and in combination with the $CD4^+CD25^-$ T cells were compared to the proliferation to the $CD4^+CD25^-$ T cells alone (set at 100%).

Flow cytometry

To determine the percentage of $CD4^+CD25^+$ Tregs in peripheral blood of the patients and healthy controls, PBMC were stained with CD4-PE-Cy5 and CD25-FITC (both from DAKO, Glostrup, Denmark). The phenotype was assessed using a combination of the following antibodies: CD152-PE, CD69-PE, CD45RO-PE (all from BD Biosciences, San Jose, USA). To stain the cells for CD152 (CTLA-4), a Fix and Perm kit of Caltag (Burlingame, USA) was used, according to the manufacturer's recommendations. Viable cells were gated based on their forward/sideward scatter profile, and analyzed using CellQuest software.

Analysis of cytokine production

The production of cytokines was measured in the supernatant of the various T cell populations upon mitogenic and antigen-specific stimulation using ELISAs specific for IL-10, IL-13, IL-5 and IFN-γ (Sanquin) according to the manufacturer's instructions. The detection limit was 1.2 pg/ml for IL-10, 0.5 pg/ml for IL-13, 5 pg/ml for IL-5 and 2 pg/ml for IFN-γ.

Statistics

The Mann Whitney test was used for statistical evaluation of the results of SPT, IgE, IgG_4 and DBPCFC before and after one year between the two different patient groups. Non-parametric paired analysis (Wilcoxon test) was performed to examine differences in the suppressive capacity of $CD4^+CD25^+$ T cells. Differences associated with p values of less than 0.05 were considered significant.

Results

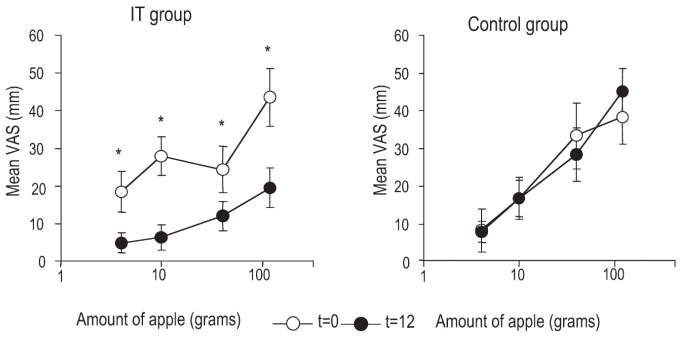
Evaluation of the clinical effect of IT with DBPCFC

All patients included in this study were challenged with fresh apple in a DBPCFC before the start of IT and after one year. All patients reacted during the initial challenges, confirming their apple allergy. At t=0 no significant differences between both groups were found. Symptoms started within 5-10 minutes after ingestion of the challenge meal, and were recognized by the patients as the typical symptoms of OAS: itching or feeling of tightness in the oral cavity or tingling of the lips. Patient reactivity was expressed using VAS scores. After one-year of IT, both groups were tested with DBPCFC. In the IT group VAS scores were significantly decreased at t=12 ($p<0.001$, Figure 1) compared to those at the start of the study. This resulted in an increase of the amount of apple tolerated of a factor of 24.

Nine of the thirteen patients treated with IT improved significantly, whereas four patients showed VAS scores similar to t=0. In three of the nine patients that showed improvement, the provocation was completely negative after one year of birch pollen IT, suggesting they had overcome their apple allergy.

In the control group, nine out of ten patients showed unchanged or even increased VAS scores relative to the year before, and no significant differences were found between t=0 and t=12. One patient in the control group was not willing to undergo a challenge for the second time. No placebos reactions were observed in both groups.

Figure 1:

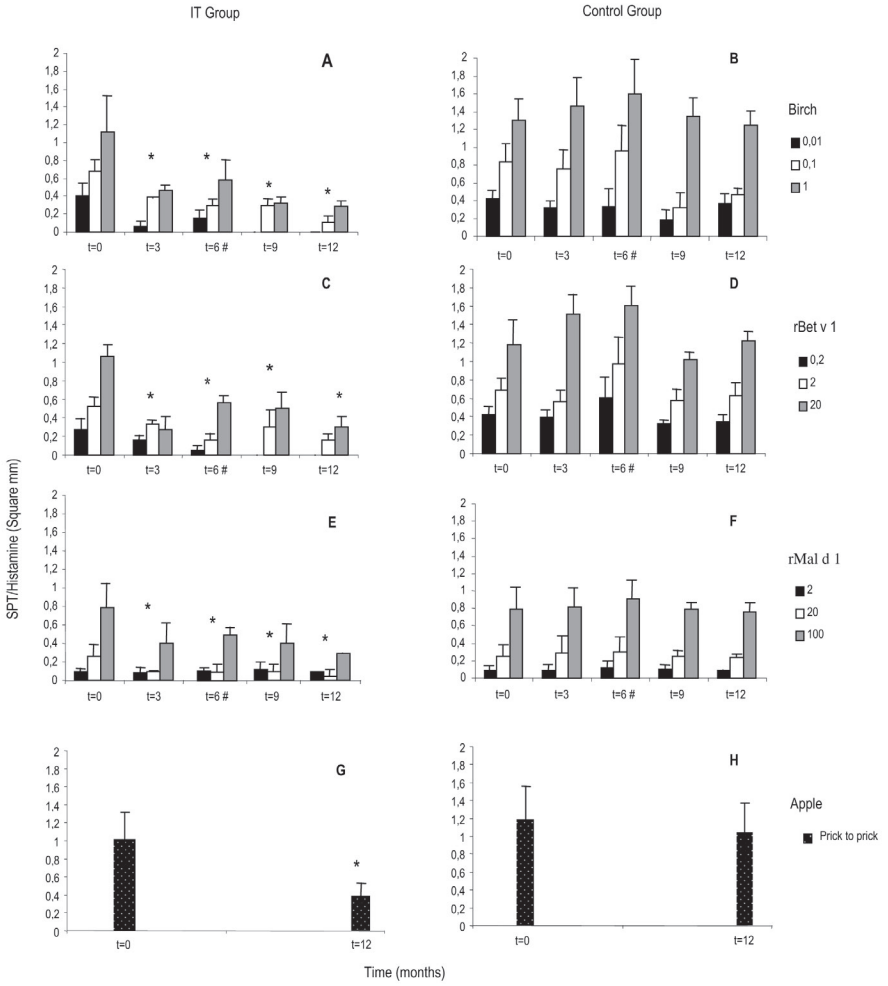


Clinical effect of IT on apple allergy measured by VAS Scores. Results are shown as mean values ± SEM for n=13 and n= 9 patients treated with (left panel) or without (right panel) IT respectively.* : $p<0.05$ (Mann Whitney test)

SPT reactivity to birch pollen, rBet v 1, apple and rMal d 1 during IT

Patients were evaluated at 3-monthly intervals by SPT with birch pollen extract, rBet v 1 and rMal d 1. For optimal accuracy, these tests were performed in serial dilutions. SPT to apple was performed at t=0 and t=12. Birch pollen IT resulted in a significant decrease of SPT reactivity to rBet v 1 and birch pollen by a factor of 30 ($p<0.001$) after 3 months of IT and drops further after one year IT by a factor 38, which was not observed in the control group (Figure 2). A 10-fold decrease ($p<0.05$) was found in SPT reactivity to rMal d 1 after three months IT and drops further after one year by a factor 20. (Figure 2, EF). Reactivity to fresh apple was also decreased significantly (Figure 2, GH).

Figure 2



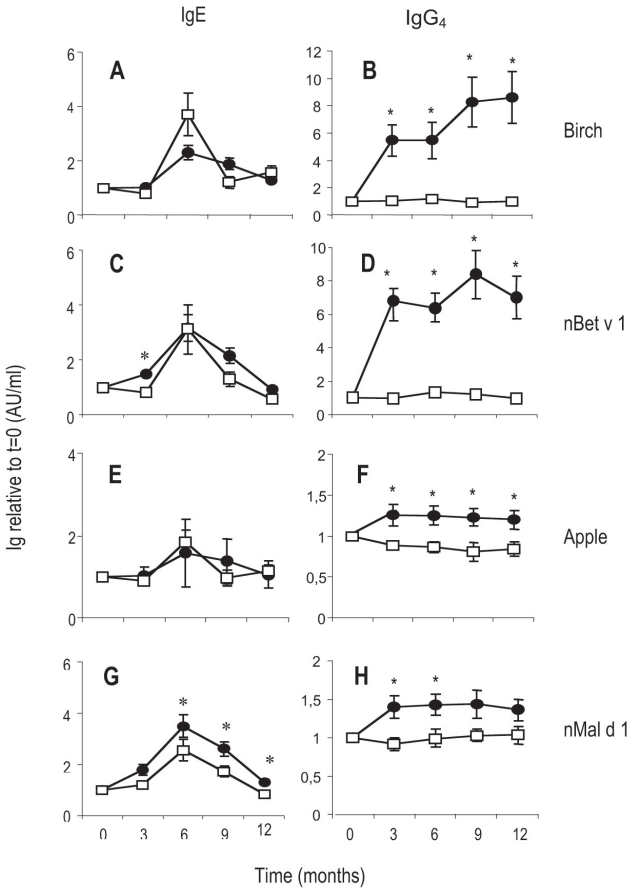
SPT reactivity to birch pollen, rBet v 1, apple and rMal d 1 in patients with birch pollen IT (A, C, E, G) and the control group (B, D, F, H). Results are shown as mean values ± SEM for n=13 and n= 10 patients treated with and without IT respectively. Significant differences compared to t=0 are shown in the Figures with an asterisk (*). Birch pollen season #.

Specific IgE and IgG_4 reactivity to birch pollen, nBet v 1, apple and nMal d 1 during IT

Specific IgE concentrations for nBet v 1 and nMal d 1 increased already before start of the pollen season in the IT group. In both groups the IgE concentrations for birch pollen, nBet v 1, apple and nMal d 1 showed an increase during the pollen season (t=6). After one year of IT, the IgE concentrations for all allergens were back to concentrations comparable to those at the start of IT in both groups (Figure 3, ACEG).

The concentration of IgG_4 for birch pollen and nBet v 1 was strongly induced in the IT group after 3 months and remained high during the total study period of one year ($p<0.05$, Figure 3, BDFH). The IgG_4 concentration for apple and nMal d 1 was also significantly elevated after 3 months in the IT group as compared to the control group ($p<0.05$). Together these data indicate that IT leads both to the induction of cross-reactive IgE as well as IgG_4.

Figure 3



Effect of birch pollen IT on birch pollen, rBet v 1, apple and nMal d1 specific IgE and IgG_4 titers. IgE and IgG_4 levels were measured via RAST and RIA. Results are shown as mean values ± SEM for n=13 and n=10 patients treated with (IT,) and without (CON,) IT respectively.* : $p<0.05$ (Mann Whitney test), t=6 pollen season.

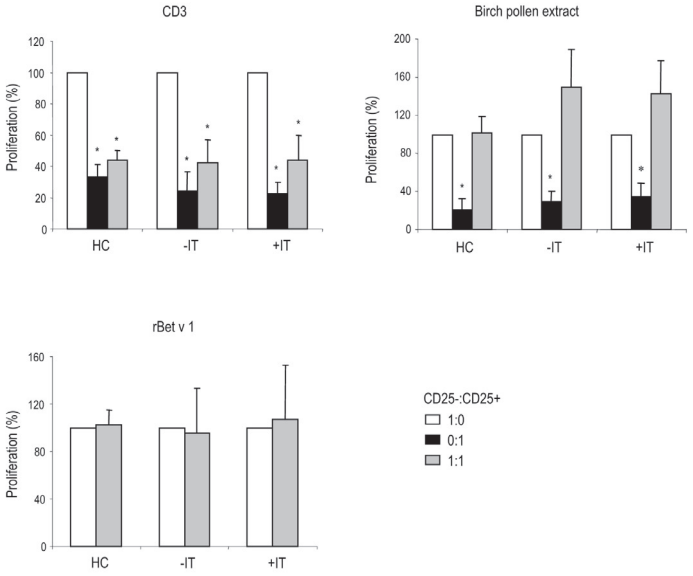
Effect of IT on $CD4^+CD25^+$ Tregs

After one year of IT treatment no differences were observed in the percentage of $CD4^+CD25^+$ Tregs in peripheral blood between IT patients, control patients and healthy controls (9.0 ± 0.7%, n=15 versus 9.16 ± 1.6%, n=10 versus 8.1 ± 0.6%, n=6, respectively). The phenotype of the $CD4^+CD25^+$ Tregs isolated from both patient groups was comparable to that of healthy controls for the expression of CD69, CD45RO and CD152 (CTLA-4) (data not shown).

$CD4^+CD25^+$ Tregs from both patient groups and from healthy controls displayed significantly lower proliferation than $CD4^+CD25^-$ T cells in response to anti-CD3 mAb stimulation or birch pollen extract, confirming the anergic state of the Tregs (Figure 4A, B). When assessing the T cell-suppressive capacity of the $CD4^+CD25^+$ Tregs (in a 1:1 ratio with $CD4^+CD25^-$ T cells), significant suppression was observed when anti-CD3 mAb was used as a stimulus (mean % inhibition 56 ± 16.4 %, 58 ± 14.5 % and 56 ± 6.2 % for IT patients, control patients and healthy controls respectively). However, in response to birch pollen extract rBetv1 or rMal d 1 in the patient groups, no $CD4^+CD25^+$ Treg-mediated suppression was observed. Also, in the healthy controls no suppression of birch pollen specific proliferation was found. The lack of suppression appeared to be specifically related to birch pollen related allergens since in both patient groups and healthy controls significant suppression of a control antigen (cow's milk antigen) by $CD4^+CD25^+$ Tregs was observed (data not shown).

For the cytokine production, addition of $CD4^+CD25^+$ Tregs to $CD4^+CD25^-$ T cells diminished the IFN-γ production after anti-CD3 stimulation in the IT patients, control patients and healthy controls. However, as for the proliferation, the IL-13, IL-5 and IFN-γ production after allergen-specific stimulation was not affected by $CD4^+CD25^+$ Tregs in any of the groups. The amount of IL-10 measured in the supernatant of the culture system was very low (5-30 pg/ml) and showed no correlation with the inhibition of proliferation by $CD4^+CD25^+$ Tregs (data not shown).

Figure 4



CD4+CD25+ Tregs derived from birch pollen allergic donors and healthy controls are not suppressive after birch pollen stimulation. CD4+CD25- and CD4+CD25+ T-cells were isolated from PBMC from healthy controls (HC) and birch pollen allergic donors with (+IT) and without birch pollen IT (-IT). CD4+CD25- (1:0 ratio, white bars) and CD4+CD25+ T-cells (0:1 ratio, black bars) were stimulated with irradiated autologous APC and the indicated antigens (CD3, Birch pollen, rBet v 1). CD4+CD25- T-cells were also cultured in presence of CD4+CD25+ T-cells (1:1 ratio, grey bars) with the same antigens. The mean proliferation ± SEM for six indepenent experiments of CD4+CD25+ T-cells and CD4+CD25+ T-cells in combination with CD4+CD25- T-cells is shown as percentages compared to the proliferation of the CD4+CD25- T cell subset alone (set at 100%). Significant differences compared to the proliferation of the CD4+CD25- T-cells are shown in the Figures with an asterisk (*).

Discussion

The current management of food allergy is based on the avoidance of the foods involved. Due to the cross reactivity of the major birch pollen allergen (Bet v 1) and many plant food allergens, patients are often allergic not only to birch, but also to various fruits, nuts and vegetables. Thus extensive elimination diets are not uncommon, which might have a social impact and may lead to vitamin deficiencies, when inadequately supervised. Therefore, there is a need for a curative treatment. This study focused at a possible role of birch pollen IT as a curative treatment for cross reactive food allergies.

Birch pollen IT induced a very rapid and significant decrease in SPT reactivity for birch pollen extract, rBet v 1 and also for apple and rMal d 1 after 3 months. Such a rapid decrease in SPT reactivity has been reported for other allergens (cat) as well[30]. At the same time, specific IgE concentrations for nBet v 1 and nMal d 1 increased, indicating the induction of a cross reactive IgE response as a result of the IT. After one year IT, SPT-reactivity was still inhibited to a similar extent as observed after three months. In contrast, IgE concentrations for all allergens were back to concentrations close to those at the start of IT, which has also been reported for other allergens[31,32]. These observations illustrate that the overall positive correlation between specific IgE and skin-reactivity is lost during IT. It is still unclear which

mechanism underlies this simultaneous increase of specific IgE titers and inhibition of skin-reactivity. Induction of specific IgG_4 antibodies is a possible factor in the inhibition of SPT-reactivity, because these antibodies were indeed observed after three months. Whether IgG_4 antibodies may be considered as blocking antibodies or just as an index of altered T-cell function remains to be determined. At least the early increase is in agreement with literature on other allergens, such as grass pollen and insect venom [33,34].

High specific IgE titers against house dust mite accompanied by poor skin-reactivity was also reported by Van de Biggelaar et al. in helminth-infected subjects [35]. The authors clearly showed that SPT-reactivity was inversely correlated with helminth-induced IL-10. An explanation for the unexpectedly low number of IgE positive subjects with a concordant skin test, as observed during chronic helminth infections and allergen-specific IT, could be the result of direct inhibition of skin mast cells by IL-10 [36] or a decrease in the number of mast cells under the influence of this inflammatory cytokine [37].

The IgG_4 response without IgE, induced by exposure to the antigen, has been called a 'modified Th2 response', as class switching to IgG_4, similar to switching to IgE, is IL-4 dependent[38] This modified Th2 response is often associated with high concentrations of IL-10, which may induce regulatory T cells. Evidence for the latter suggestion came from a mouse model where induction of tolerance led to IL-10-secreting dendritic cells and induction of regulatory T cells [39], which suggests a key role for regulatory T cells in the development of tolerance induction.

Therefore, the effect of IT on the number and function of the regulatory $CD4^+CD25^+$ T cell population was examined in both patient groups. The data in this study show that their number was not enhanced in peripheral blood of patients receiving IT. For short-term birch pollen IT it was shown that IT could modulate antigen-specific T cells at local sites (in the nose) but not in the periphery [40]. Therefore, although their presence in the local tissues (lung, nose, lymph nodes) was not investigated in this study, the $CD4^+CD25^+$ Tregs may be enhanced in their number at local sites as a result of IT. The proliferative T cell response to anti-CD3 mAb and cow's milk antigen was clearly suppressed by addition of the $CD4^+CD25^+$ Tregs in both patients groups. Suppression was not observed when crude birch pollen extract or the recombinant allergens (rBet v 1 and rMal d 1) were used. Previously, we demonstrated that in cow's milk allergic individuals and in healthy controls the cow's milk-specific T cell response can be suppressed by $CD4^+CD25^+$ Tregs [41]. The lack of suppression by $CD4^+CD25^+$ Tregs towards birch pollen observed in this study may reflect differences in either recognition or presentation of the allergen. These differences are not related to the allergic status of an individual, since suppression was also absent in the healthy controls. Akdis *et al* previously proposed that the $CD4^+CD25^+$ Tregs are the source of the produced IL-10 in a bee venom-specific immunotherapy trial [11]. However, whether the suppression of an allergen-specific T cell response by $CD4^+CD25^+$ Tregs is mediated via IL-10 remains unclear [42]. Our data and the results from others do not demonstrate an increase of IL-10 production in culture supernatant after successful birch pollen IT [43]. Still, analysis of the IL-10-producing capacity of cells with intracellular staining after IT could perhaps have resulted in higher values in the IT group, as this has recently been shown in a study with grass pollen IT [44]. Together, these findings indicate that birch pollen-IT did not result in an induction of the suppressive function of the $CD4^+CD25^+$ Tregs towards the T cell response towards birch pollen, rBet v 1 or rMal d 1 and was also not associated with higher concentrations of IL-10 in the supernatant.

So far, the limited number of studies investigating the clinical effects of pollen IT on food allergy were not optimally controlled and gave conflicting results [16-19].

For another inhalant-food allergen crossreactive syndrome, namely mite-snail allergy a harmful effect of IT has been described [45]. So possible effects of IT with inhalant allergens on the related food allergy can not be excluded. This might be related to the allergen involved. In order to focus on the active molecules of this cross-reactive allergic syndrome, we chose to use purified natural and recombinant allergens as reagents. These molecules proved to be very sensitive and stable tools for measuring changes in humoral immune responses and in SPT reactivity. For assessment of clinical food allergy the DBPCFC is generally regarded as the most reliable technique. Earlier studies were limited to recording clinical histories or open food challenges. Our study is the first clinical trial that has performed DBPCFCs to monitor clinical reactivity to apple. To avoid breakdown of the labile allergen Mal d 1, each active dose was prepared within 5 minutes prior to administration. This protocol facilitated the use of labile food allergens in DBPCFC. Of course real proof for the clinical effect has to come from a trial in which not only the evaluation is blinded as we did, but also the IT treatment is placebo-controlled. Such study is now under preparation. Despite the lack of a placebo treatment group, the challenge data appear to support a clinical effect of the treatment. Challenge data of the control group did not significantly differ at t=0 and t=12, indicating a good reproducibility of the method. A recent study performed by our group had already demonstrated that threshold dose in peanut and hazelnut allergic patients determined by DBPCFC do not significantly change over time [46].

In conclusion, this clinical trial provides support for earlier reports claiming that birch pollen IT has a beneficial effect on cross-reactive food allergies. Furthermore, our study has established the use of recombinant allergens and DBPCFC as tools for monitoring cross-desensitization in future double-blind placebo-controlled studies.

Acknowledgements

This study was supported by a grant from the EC: SAFE QLK1-CT-2000-01394. We thank ALK –ABELLÓ for financial support of part of this study. We acknowledge Dr. M. Fernandez-Rivas for valuable advice concerning the recipe for DBPCFC. Furthermore, we thank Adrie van Ieperen-Van Dijk and Inge de Vegt-Krikken for excellent technical assistance.

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9

A mutant of the major apple allergen, Mal d 1 demonstrating hypo-allergenicity in target organ by DBPCFC

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Submitted

Abstract

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

Objective: To demonstrate hypoallergenicity 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. Hypoallergenicity of the mutant rMal d 1(rMal d 1 mut) compared to 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 (SPT) and double-blind placebo-controlled food challenge (DBPCFC).

Results: RAST analysis (n=14) revealed that IgE-reactivity to rMal d 1mut was two-fold lower than to the wild-type molecule (95% 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 to 200-fold. Hypoallergenicity 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 hypoallergenic. This opens the way to safer immunotherapy for the treatment of food allergic patients.

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 *et al.*[3] and is now widely used as an effective treatment of 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 *et al.* 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 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 hypoallergenic 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]. Hypoallergenicity was evaluated by *in vitro* and *in vivo* methods (SPT). Only in one study, hypoallergenicity 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 were not assessed for hypoallergenicity by *in vivo* methods like SPT and DBPCFC. One of the main reasons for this reluctance is the remaining fear of anaphylactic reactions. In this study, therefore, we chose to assess the general feasibility of the hypoallergen-approach by mutating the 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.

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 mm^2) and/or a positive RAST (≥ 0.7 IU/ml) for birch pollen and apple. All patients had seasonal rhinitis in the period of February-April, being 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[17]. Recombinant (r)Mal d 1 (EMBL Access no: AJ417551) was produced in *E. coli* and purified as described elsewhere (manuscript submitted). A hypoallergenic mutant thereof, carrying 5 point mutations, designated rMal d 1mut, was designed and produced by analogy to hypoallergenic Bet v 1mut.[18] rMal d 1mut was expressed in E. coli and purified to homogeneity (manuscript submitted).

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[19]. 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 minutes by copying the wheal reaction with transparent adhesive tape onto a record sheet for later comparison. The skin wheal areas were evaluated by computer scanning[20].

Basophil histamine release assay (BHR)

White blood cells were isolated from blood of a non-allergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere[21,22]. 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 microgram/ml). Liberated histamine was measured by the fluorometric method essentially as described by Siraganian[23]. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Centre under project number MEC 97/030.

RAST and RAST 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[24,25]. Briefly, serum (50 µl) was incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 µl PBS, 0.3% BSA, 0.1% Tween-20 (PBS-AT). After washing away unbound serum components, radiolabeled sheep antibodies directed to human IgE were added to a final volume of 500 µl in PBS-AT plus 4.5% bovine and 0.5% sheep serum (v/v) (Sanquin, Amsterdam, The Netherlands). After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units IgE per ml (IU/ml). Calculation was performed by means of a standard curve that was obtained by RAST with a dilution series of a chimeric

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

Ptno	Sex	Age (yr)	Apple related symptoms (CH)	SPT Apple (mm^2)	SPT Birch (mm^2)	RAST Birch (IU/ml)	RAST Apple (IU/ml)	SPT rMal d 1 2 ug/ml (mm^2)	SPT rMal d 1mut 2 ug/ml (mm^2)	Ratio 2 ug/ml (mm^2) SPT	RAST rMal d 1 (IU/ml)	RAST rMal d 1mut (IU/ml)	Ratio RAST
1	F	18	OAS RC CU	52	49	12,3	5,8	7	Neg	7	0,3	0,2	1,6
2	F	28	OAS	42	23	5,8	3,0	*	Neg*	6	0,9	0,7	1,3
3	F	30	OAS RC CU	64	25	11,4	4,8	24	Neg	6	2,6	1,3	2,0
4	F	30	OAS	30	44	3,4	1,3	20	Neg	20	1,1	0,4	3,1
5	M	46	OAS	49	25	11,7	4,8	8	Neg	8	2,2	1,4	1,5
6	F	44	OAS RC	22	43	23,8	8,2	13	8	2	0,4	0,1	2,9
7	F	35	OAS RC	48	35	14,1	6,8	*	Neg*	2	5,7	2,5	2,3
8	M	37	OAS	30	29	3,4	1,1	12	Neg	12	0,3	0,2	1,4
9	M	45	OAS RC CU	55	10	2,9	2,2	12	Neg	12	0,9	0,7	1,2
10	M	50	OAS	15	38	5,8	2,7	11	Neg	11	0,3	0,1	2,3
11	F	44	OAS RC	29	20	17,8	10,0	13	Neg	3	5,4	3,2	1,7
12	F	22	OAS RC CU	42	33	26,1	5,3	26	Neg	7	0,5	0,2	3,3
13	F	48	OAS RC	20	33	4,6	1,8	35	Neg	35	0,7	0,4	1,9
14	M	31	OAS RC	64	29	32,3	18,8	24	Neg	5	13,7	7,8	1,8

OAS, Oral Allergy Syndrome
RC, Rhinoconjunctivitis
CU, Contact Urticaria

monoclonal IgE antibody against the major house dust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2[26]. A RAST value >0.3 IU/ml was regarded positive.

For RAST inhibition, serum was pre-incubated with serial dilutions 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. Subsequent steps were identical to those described for the RAST. Results were expressed as percentage inhibition.

SDS-PAGE/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, California, USA) and silver stained using a protein silver staining kit (Bexel Biotechnology, Union City, CA, USA).

Western blotting was performed by semi-dry transfer to 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 minutes, blots were cut into strips prior to immunoprobing overnight with 150 µl human serum in 3 ml of PBS-AT. After washing away unbound serum with PBS/ 0.1% Tween-20, radiolabeled sheep antibodies against human IgE were used for detection of bound IgE. Blots were exposed to X-ray film (Eastman Kodak Company, Rochester, NY, USA). For blot inhibition studies, 150 µl of the inhibitor was added in several concentrations together with the patient's serum. Incubation of the blotstrips and detection were performed as described above.

DBPCFC

Clinical reactivity of both the rMal d 1 and its mutant was investigated by DBPCFC in two patients using a procedure, which has been described before[27]. In short, on the day of the challenge only a light breakfast was allowed at the latest 2 hours 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 permanently 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, blood pressure, heart rate and peak flow were measured for safety reasons and as parameters for detecting allergic reactions. An interval of 15 minutes was taken between each test meal, or as long as was needed for (subjective) symptoms to disappear. Patients were kept for observation for at least one hour after resolution of symptoms. The severity of the oral allergy symptoms, was scored using visual analogue scales (VAS) ranging from 0-100 mm, where 0 equals no symptoms and 100 stands for severe symptoms.

The challenge drinks for provocation with recombinant allergens consisted 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 done to confirm clinical reactivity to apple. Each challenge meal with fresh apple was prepared within five minutes before administration and contained: 4, 10, 40 or 120 grams 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 due to processing steps and were added for optimal blinding of taste[28].

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:

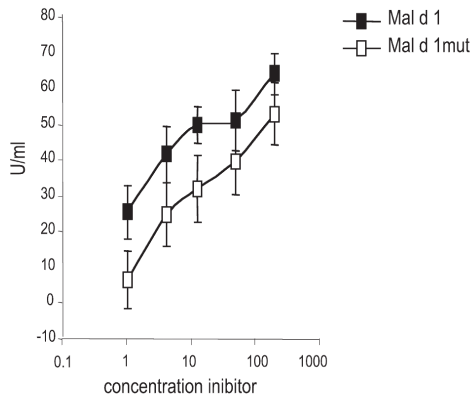
IgE binding potency

All 14 patients were tested by RAST with rMal d 1 wild type and its mutant, rMal d 1mut. Mean specific IgE to rMal d 1 was 2.5 IU/ml ranging from 0.3 to 13.7 IU/ml. Mean specific IgE to rMal d 1mut was 1.4 IU/ml ranging from 0.1-7.8 IU/ml. The mean ratio of IgE reactivity rMal d 1mut and rMal d 1 was 0.5 (95% CI: 0.4-0.6).

Six sera were used for RAST inhibition with rMal d 1 and rMal d 1mut. The IgE binding potency was decreased by a factor 7.8 (95% CI: 3.0-12.6) in RAST inhibition (Figure 1).

All sera were analyzed by immunoblot with rMal d 1 and rMal d 1mut. By densitometric scanning, it was found that IgE reactivity to the rMal d 1mut was decreased by 25-45% compared to the binding to the rMal d 1 (results not shown).

Figure 1: RAST inhibition comparison of rMal d 1 and rMal d 1 mut



In vivo (SPT) and in vitro (BHR) biological activity

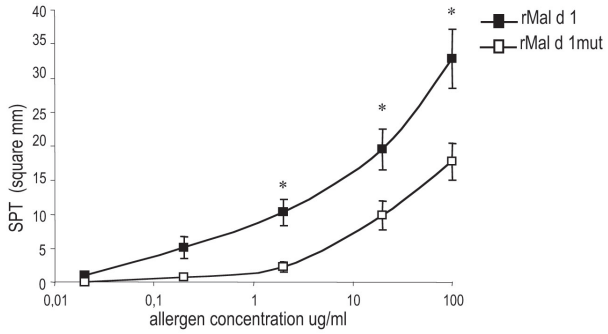
rMal d 1 and rMal d 1mut were tested by titrated SPT in 14 patients. Parallel-line analysis showed that the mutant molecule had a 10-fold lower biological activity than the wild-type molecule. In concordance with this observation the lowest concentration giving a positive SPT (>7 mm^2) was 2 μg/ml for rMal d 1 and 20 μg/ml for rMal d 1mut (Figure 2).

Serum of three patients (3,11 and 14) was used to sensitise stripped basophils. Upon incubation with either rMal d 1 or rMal d 1mut, the former molecule demonstrated a 95, 90 and 163-fold lower biological reactivity, respectively (Figure 3). Compared to 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.

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

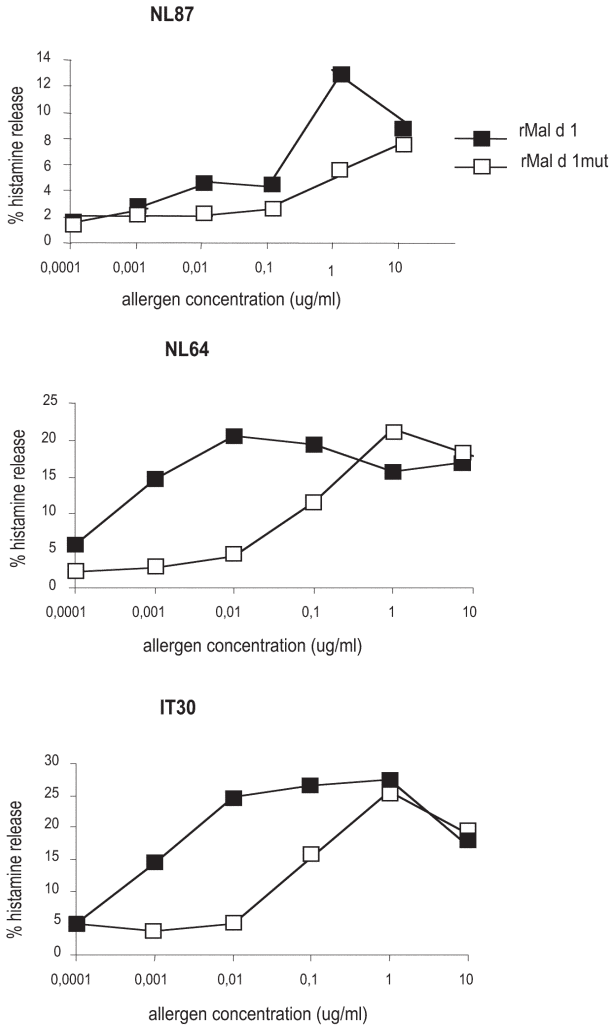
Patient nr.		**3**	**11**	**14**
		Factor	**Factor**	**Factor**
SPT	rMal d 1 vs. rMal d 1mut (1st reaction > HEIC 0.25)	100	100	100
BHR		95	163	90
RAST inhibition		2.8	7.9	4.6
RAST		2.0	1.7	1.8
Blotscan		1.4	1.5	1.8

Figure 2: SPT reactivity to rMal d 1 and its mutant



Results are shown as mean values ± SEM for n=14 patients. * : $p<0.05$ (paired t-test)

Figure 3: Basophil histamine release.



Comparison biological activity of rMal d 1 and rMal d 1 mut

Difference in clinical reactivity of rMal d 1 and rMal d 1mut as determined by DBPCFC

Two patients (no. 1 and 2) underwent a DBPCFC with three ten fold increasing doses of rMal d 1 and its mutant (Table 2). Both patients had a positive DBPCFC with fresh apple (Golden Delicious).

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 minutes 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 occurred at the higher dose of 1 mg. Reactivity to rMal d 1mut only occurred at the highest concentration tested (1 mg) (Figure 4). No placebo reactions occurred.

Patient 2 reported typical oral allergy symptoms within 5 minutes 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 dyspnoe (drop of 50% of peak-flow) and the patient was treated with inhaled $ß_2$-mimetic, oral antihistamines, and oral Prednison 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 dyspnoe were observed (Figure 4). No placebo reactions were reported.

Table 2: DBPCFC with rMal d 1 and its mutant patient 1 and patient 2

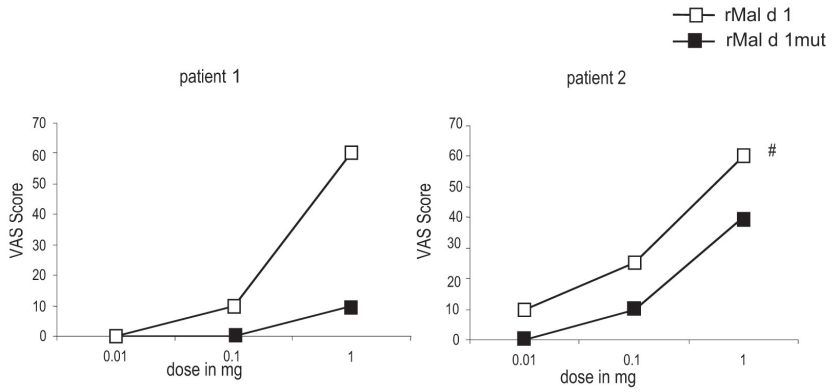
Patient 1

Allergens	Dose in mg	Reactions
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

Allergens	Dose in mg	Reactions
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

Figure 4: DBPCFC rMal d 1 and rMal d 1mut.



Hoarsness, Coughing, Dyspnoe with 50% peak flow increasing

Discussion

This is the first study demonstrating by DBPCFC and SPT that a recombinant food allergen can be made that is clinically hypoallergenic. By mutating just 5 surface-exposed aminoacids 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 hypoallergenic peanut allergens can be hyperallergenic 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 hypoallergenic recombinant molecules is considered. Before the start of such a treatment, hypoallergenicity should therefore always be confirmed on an individual basis. The stripped basophil test for the assessment of biological activity has proven to be a very sensitive tool that could be used as a precautionary test prior to the start of treatment with hypoallergens. Probably SPT with hypoallergens involved will also give valuable information.

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

An unresolved question is how hypoallergenic 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 a.o. 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 variation in their threshold at minimum provoking dose. These have been studied for several foods like hazelnut, peanut, milk and egg[27,29,30]. Thresholds roughly ranged from 10 µg to 100 mg of these foods. In a

food like peanut the major allergens are storage proteins that make up a large part of the total protein. This implies that threshold doses for individual peanut allergens most likely lie in the µg to mg 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 hypoallergenic major allergen was well tolerated, and 1 mg gave only mild symptoms. In contrast a quantity of 1 mg of the wild-type allergen gave quite severe symptoms in one of these patients. 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[29]. To be able to safely administer milligram quantities of major peanut allergen to patients with severe peanut allergy, most likely a larger reduction of allergenicity is required than now achieved for the major apple allergen.

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

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10 General discussion

Sensitization and cross-reactivity

Our studies in four European countries have once more confirmed that the presence of birch trees is a dominant factor in sensitization to *Malus domestica* (Mal d) 1. Primary sensitization to birch pollen is at the basis of immunoglobuline E (IgE) recognition of Mal d 1 in Northern and Central Europe[1]. In Madrid where birch trees are virtually absent, the mean IgE response to Mal d 1 was low: 0.7 IU/ml. In contrast, this value was 4-6 times higher in areas endemic for birch trees: 2.8 IU/ml in The Netherlands, 4.4 IU/ml in Austria and 3.7 IU/ml in Northern Italy. These differences were even more significant for IgE responses to the primary sensitizer *Betula verrucosa* 1 (Bet v 1): 0.8 IU/ml versus 23.3, 39.9 and 27.0 IU/ml, respectively. Nevertheless, 55% of the patients from the environs of Madrid had a low but positive radio allergosorbent test (RAST) (> 0.3 IU/ml) for Mal d 1. Only 27% had a weak positive RAST to Bet v 1, and the mean ratio of IgE to Bet v 1 over Mal d 1 was 0.7. This is not in line with notion that primary sensitization to Bet v 1 is a prerequisite for sensitization to Mal d 1[2,3]. In the areas where birch pollen is abundant (The Netherlands, Austria and Italy), the ratio of IgE to Bet v 1 over Mal d 1 was >10. The pathway of sensitization to Mal d 1 and Bet v 1 in Madrid is still unclear. The fact that IgE titers to Mal d 1 were slightly higher than to Bet v 1 indicates that Bet v 1 can not be the primary sensitizer causing cross-reactivity to Mal d 1[4]. At the same time, the extreme lability of Mal d 1 argues against direct sensitization by apple consumption. A more plausible explanation would be that sensitization to pathogenesis-related (PR) 10 proteins is induced by other pollen containing an allergenic PR10 protein. Obvious candidates are alder and hazel[5] but their pollen are also not present in significant quantities in the vicinity of Madrid (www.polleninfo.org). The main representative of the order of the *Fagales* trees responsible for significant pollen counts in that area is oak (*Quercus alba*)[6,7]. Further studies will have to demonstrate whether primary sensitization to food homologs of Bet v 1 or to another source of pollen PR10 protein is at the basis of anti-Mal d 1 IgE in birch pollen free areas.

Cross-reactivity and severity of symptoms

Our studies have demonstrated that a small minority of patients with combined birch pollen-food allergy can develop more severe systemic reactions. This was true for both the jackfruit and the sharonfruit allergic patients described in Chapters 3 and 4, respectively[8]. Bet v 1-specific IgE antibodies played a dominant role in cross-reactivity in these patients as well. Profilin (Bet v 2) and Bet v 6 were also involved in cross-reactivity, but these allergens probably had limited clinical relevance as judged by histamine release tests. On the basis of these observations we conclude that Bet v 1 homologs in jackfruit and sharonfruit played an important role in the more severe symptoms suffered by these patients. This is not in line with the general view that these allergens are too labile to be able to induce systemic symptoms[9,10]. Nevertheless, claims that PR10 proteins in food can induce severe food allergy have been made by Kleine-Tebbe *et al.*, i.e. for *Glycine max* (Gly m) 4, the Bet v 1 homolog in soy[11]. Inhibition assays demonstrated that primary sensitization to Bet v 1 was at the basis of IgE recognition of Gly m 4. For some of the patients with anaphylactic reactions to a soy drink, Gly m 4 was the only allergen recognized on soy immunoblot and it was

therefore concluded that Gly m 4 induced anaphylaxis. The authors did not really provide an explanation as to how a presumably labile allergen like Gly m 4 was capable of inducing systemic reactions. A more recent publication showed that Gly m 4 is resistant to moderate heating. The allergen was still detectable in soy after two hours of cooking[12]. Similarly, Vieths *et al.* and Ballmer-Weber *et al.*[13,14] showed in double-blind, placebo controlled food challenge (DBPCFC) studies that both cooked celery and roasted hazelnut caused allergic symptoms in patients apparently monosensitized to PR10 allergens. Increased resistance of these Bet v 1 homologs to proteolytic attack has however so far not been demonstrated. Although confirmatory data are still missing, there appear to be differences in the stability profiles of Bet v 1-related allergens. How can these differences be explained? One explanation might be that the matrix provided by e.g. soy to Gly m 4 protects it from the influence of heating and/or proteolysis. Very little is known about matrix effects on the stability of food allergens. Grimshaw *et al.*[15] recently showed by DBPCFC in a small number of patients that the fat content of a challenge vehicle can have a profound effect on the allergic reaction. They reported that peanut allergic patients consumed larger doses of peanut before they first noticed a reaction when the fat content of the vehicle was higher. The reason for this was that they did not experience oral symptoms as "early warning signs". The actual allergic reaction was however more severe and systemic. The interpretation of the authors was that allergens in a high-fat food matrix are released more slowly than in a low-fat matrix, thus preventing oral symptoms. Perhaps such a mechanism could also protect otherwise labile Bet v 1 homologs. For soy, a high-fat content might play a role in the protection of Gly m 4, but for sharonfruit and jackfruit this explanation is less plausible.

An alternative explanation would be that certain PR10 molecules have increased stability as a result of differences in their primary structure. Even point mutations can have very significant effects on the stability of proteins[16]. As yet, there is however no experimental proof to support this hypothesis for PR10 allergens. A completely different explanation should therefore perhaps still be considered. It is very possible that the food allergy observed in these birch pollen allergic patients is totally unrelated to IgE antibodies against Bet v 1 homologs like Gly m 4, but against other so far unknown more stable allergens. Food extracts used in RAST- or immunoblot-inhibition might not have contained all relevant allergens because e.g. some food allergens might not easily extract under the condition normally used or because allergens get depleted from extracts during defatting steps. An allergen that is not adequately presented on the solid phase of a RAST or on immunoblot can not play a role in competitive assays with Bet v 1 as inhibitor. The complete inhibition of IgE binding to such incomplete food extract by Bet v 1 would then be mis-interpreted: complete inhibition is only observed because IgE antibodies against the missing allergen(s) went undetected. Further studies are needed to establish whether relevant allergens are missing or PR10 proteins can sometimes indeed induce severe reactions.

Cross-reactivity and novel foods

Cross-reactivity and sequence homology are key issues for legislation aiming at regulating entry of novel foods to the market[17]. The discussion has so far mainly focused on genetically modified foods but is not limited to those biotech products. Sequence homology to known allergens is one of the main criteria by which to judge whether a protein can be introduced

into a food or not. Cross-reactivity as a result of sequence homology is very well-established for non-transgenic plant foods that are already on the market. In chapters 3 and 4 two tropical fruits (jackfruit and sharonfruit) that are relatively novel on the European market were shown to induce allergic reactions on the basis of cross-reactivity to birch pollen. Increased consumption will certainly result in a higher incidence of adverse reactions. This is in line with a recent study in Sweden[18] where 397 birch pollen allergic patients were asked to fill out a questionnaire about possible reactions to a number of exotic fruits that are not common in that area. Of 112 patients who had eaten sharonfruit, 19 (17 %) reported an allergic reaction. Whether current legislation would still allow introduction of such fruits to the market is an interesting question. Is the potential allergic reaction of some people sufficient reason to deny the rest of the population access to tasty fruits or nuts? Strict adherence to current legislation would most likely mean that fruit with a high nutritional value like kiwi would not be allowed to enter the market today as it did some decades ago. A recent example of a novel food illustrates that this question is not absurd. There was talk of banning from the market a tropical nut from Micronesia, the Nangai nut was proposed to be banned from the market after it was shown to induce allergic responses in some pollen-allergic patients[19].

Why is lipid-transfer- protein a Mediterranean allergen?

While the absence of Bet v 1-related fruit allergies in Spain has a straightforward explanation (i.e. the absence of birch tress), it is most difficult to explain why sensitization to lipid transfer protein (LTP) almost exclusively occurs in Mediterranean countries. In part of southern Europe allergies to apple and related *Rosaceae* fruits such as peach and plum are mainly caused by LTP[4,20-22]. Symptoms tend to be more severe and patients do not always have concomitant pollen allergies. The general perception is that LTP sensitization occurs directly through fruit consumption, i.e. independently of pollen sensitization[4,21]. It is thought that the extreme stability of LTP facilitates both its role as a sensitizing food allergen and as an allergen linked to severe anaphylaxis[4,23-25]. If LTP in fruits is responsible for sensitization in Spanish fruit allergic patients, why then does fruit consumption in Northern and Central Europe not leading to LTP sensitization? Is fruit consumption in general lower in these areas? True consumption Figures are hard to obtain. Some databases contain per capita supply Figures that are obtained by analyzing local production and import and export Figures. The Food and Agriculture Organization (FAO) database lists per capita supplies for most countries in the world (www.fao.org/faostat). From this database no evidence is found for significantly higher overall fruit consumption per capita in Spain compared to the other three countries in our survey: 125.6 kg/year in Austria, 139.9 kg/year in Italy, 129.3 kg/year in The Netherlands versus 122.6 kg/year in Spain for the year 2001. Are specific fruits perhaps more commonly eaten in Spain? The FAO data for apple do not point in that direction with Spain having the lowest Figure of 18.4 kg/year per capita compared to 48.4 for Austria, 22.6 for Italy and 34.3 for The Netherlands. Unfortunately this database does not list these Figures for peach. Peach allergy is the most dominant fruit allergy linked to LTP sensitization[22]. Fresh peaches are available for a much longer period of the year in Spain than in more temperate climates. RAST Figures for peach were significantly higher in Spain than in the other three countries which might indeed point towards higher exposure. Reliable consumption Figures

are urgently needed to find out whether differences in sensitization to peach and to LTP are indeed explained by differences in exposure. Our study has also revealed that the onset of fruit allergy occurred at a younger age in Spain. To study whether peach is introduced at a younger age in Spain than in the other countries we performed a survey in supermarkets, analyzing the presence of peach in ready-made food products for babies and toddlers. The chance of finding peach in these products was indeed significantly higher in Spain than in the other three countries. All together, these data provide some support for the idea that LTP sensitization does not occur in Northern and Central Europe because consumption of peach is lower and starts later in life.

Despite the fact that LTP sensitization does occur in patients without known pollinosis, a possible role for primary sensitization should not be completely ruled out. Various weeds and trees have now been shown to express LTPs in their pollen[26-28]. The major allergens from *Parietaria judaica* (Par j) 1 and 2, have been identified as LTPs[29]. There is however no evidence supporting cross-reactivity to fruit LTPs. A limited degree of cross-reactivity has been demonstrated between LTP from mugwort pollen and from fruits, but the data certainly did not support primary sensitization to mugwort as the cause of LTP sensitization. Another pollen that has been linked to LTP sensitization and allergy to apple, peach, hazelnut, chickpea, peanut, lettuce and maize is plane pollen (*Platanus acerifolia*)[30]. The homology of plane pollen LTP and fruit- and nut-LTP is unknown. Interestingly, high exposure to plane pollen in Europe is concentrated in central and eastern Spain (www.polleninfo.org). A role for exposure to specific pollen such as from plane should therefore not be completely ruled out as (a partial) explanation for the differences observed in sensitization to LTP across Europe.

Improved diagnosis: application of purified allergens

The gold standard of food allergy is still the (laborious and burdensome) DBPCFC[31]. Skin prick test (SPT) for fruits using commercial extracts have very poor sensitivity due to the lability of many fruit allergens[32,33]. The alternative prick-to-prick method is far much sensitive but is not easily standardized and is dependent on the availability of fresh fruits[34,35]. *In vitro* IgE tests usually have much higher sensitivity than SPT but often have poor specificity. False-positive CAP or RAST are not uncommon[36-38]. Well-known examples of specific IgE antibodies that usually lack biological activity (i.e. clinical relevance) are IgE antibodies directed to cross-reactive carbohydrate determinants (CCD) or profilin[39-44]. Moreover, a true positive *in vitro* test using food extracts does not provide information on the potential severity of the reaction that can be expected. Our study on apple allergy performed across Europe has demonstrated that application of individual apple allergens is a powerful tool to distinguish between IgE responses with a different risk profile. If a patient has a positive RAST for Mal d 3, LTP, the patient should be advised to avoid the fruit because LTP can cause severe systemic reactions. If, however, apple-specific IgE is directed to Mal d 1 there is a negligible safety risk. For important allergenic foods like peanut similar strategies can be applied. True peanut allergies with potentially life-threatening consequences are linked to allergens like Ara h (*Arachis hypogea*) 1, 2 and 3[45]. If peanut-specific IgE is directed to pollen-related peanut allergens, like Ara h 8 (D. Mittag, J. Akkerdaas, unpublished data) there is little or no of severe reactions. The application of well-defined purified (natural

or recombinant) food allergens is therefore a major step forward for *in vitro* food allergy diagnostics[38]. Such reagents will also prove to be useful in SPT, not in the last place because purified allergens like for example Mal d 1 are stable in contrast to commercial apple extracts.

Treatment: avoidance of fruits and nuts

The current management of food allergy is based on the avoidance of the foods involved, and if necessary rescue medication. Since Bet v 1-related fruit allergies are probably never life-threatening and only inconvenient, some patients decide to continue eating apples and related fruits and nuts. Most patients will however try to avoid allergic reactions whenever possible, and the high degree of cross-reactivity then means eliminating a rather broad spectrum of fruits and nuts will from their diet. These patients will deprive themselves of important sources of vitamins, minerals and fibres. For patients with LTP-induced fruit allergy avoidance can be a matter of life and death and the impact on their quality of life is much greater. Therefore, there is a need for alternative strategies for treatment.

Treatment: naturally occurring hypoallergenic fruits

Another possibility for the treatment of food allergy is to search for hypoallergenic varieties of foods. In that way diets could perhaps become less restrictive. Apple allergic patients sometimes report that they tolerate some specific cultivars better than others. A number of publications have indeed confirmed differences in allergen activity between cultivars, mainly using *in vitro* test systems[46-48]. Vieths *et al.*[46] used extracts of apple for *in vivo* assessment by SPT. We set out to assess the allergenicity of a spectrum of cultivars by the prick-to-prick test for initial screening and by DBPCFC for final confirmation. In this way we circumvented the problem of loss in allergenicity of Mal d 1 observed in extracts used for SPT[49,50]. Indeed, significant differences in allergenicity were confirmed by SPT and DBPCFC. For patients with mild Mal d 1-induced oral allergy such information is useful, allowing them to eat apples with reduced allergenicity. Although there were some individual differences between patients, the overall ranking of cultivars allowed a distinction to be made between apples of high and low allergenicity. The value of this analysis has its limitations because the outcome is most likely of little relevance for patients from Spain with Mal d 3-induced apple allergy. A limited SPT analysis performed with three apple cultivars in a group of Spanish and a group of Dutch patients indeed showed that the ranking was not identical. In other words, hypoallergenic with respect to Mal d 1 is not the same as hypoallergenic with respect to LTP. Taking the risk of severe anaphylaxis into account, identification of hypoallergenic cultivars is of little use anyway to LTP patients who need to follow a regimen of complete avoidance.

Both Mal d 1 and Mal d 3 are members of the PR family[51]. PR-proteins can be induced upon pathogen exposure or physical stress like bruising. There are several occasions along the route from apple tree to consumption when such stress can occur. Growing conditions (e.g. organic versus conventional), harvest, transport and storage can potentially all influence the level of these PR-proteins and consequently allergenicity. Our SPT studies revealed that

allergenicity increased during storage and that reducing oxygen in the cool storage room could partly prevent this increase. Again these studies were performed in Mal d 1-sensitized patients and it is not known whether LTP behaves similarly. Nevertheless, it appears to be possible to decrease allergenicity by controlling storage conditions.

Treatment: biotech hypoallergenic foods

An alternative manner to obtaining hypoallergenic foods is to inhibit expression of allergens by molecular biological techniques like RNA interference[52]. So far only the production of hypoallergenic rice has been reported, but its hypoallergenicity has only been assessed by *in vitro* methods like immunoblot and enzyme-linked immunosorbent-assay (ELISA)[53]. In our studies the successful inhibition of Mal d 1 expression was demonstrated by both *in vitro* and *in vivo* methods. The time frame of our project did not allow us to assess the allergenicity of fruits, but we knew from pilot experiments that Mal d 1 is also expressed in leaves. This allowed us to analyse small shoots of apple trees for allergenicity. Our experiments have demonstrated the technical feasibility of producing biotech hypoallergenic apples, although the stability of inhibition of expression in off-spring still has to be assessed. Available data from gene silencing in *Arabidopsis* show stability for at least 5 generations[54].

Public opinion on genetically modified foods has proven to be perhaps a bigger hurdle. The appearance of genetically modified foods in the marketplace has resulted in an emotional and fierce public debate, scientific discussion, and media coverage. New advances made possible by genetic modification met with a variety of ecological and human health concerns[55]. Most genetically modified foods that have reached the market so far were designed to give higher production yields at lower cost. Whether biotech foods with a consumer benefit like hypoallergenicity would more readily be accepted by the general public or more specifically by allergic consumers is not really known. To address this question food allergic consumers were interviewed to assess their attitude towards low allergen GM foods. The results suggested that food allergic consumers are interested in having access to low allergen foods, with 70-95% of the sample wanting low allergy food to be produced. Participants in the study identified a number of benefits to themselves, such as being able to eat the food again to which they are allergic, and being able to eat all food without worries, no symptoms and no need to check labels. But what is the advantage of hypoallergenic food for non-allergic persons? Non-allergic consumers of course have other priorities and would refrain from buying hypoallergenic foods if the price was much higher or if they were less tasty. Most likely, the commercial success of hypoallergenic foods will depend on their attracting more than just allergic consumers.

Immunotherapy with cross-reactive allergens

Of course the ideal treatment for food allergy would be to cure the disease. One of the therapeutic possibilities that comes closest to a cure is allergen-specific immunotherapy (IT). IT for the treatment of pollen allergy has been a well-accepted and successful therapy for many years[56]. In view of the cross-reactivity between birch pollen and fruits and/or nuts, IT with birch pollen allergens might be expected to improve associated food allergies. This

has been studied by different groups but results are conflicting[57-60]. Some studies reported improvement[57,58,60], while others found no beneficial effect of tree pollen IT on apple allergy[59,60]. In chapter 8 we evaluated the clinical efficacy of birch pollen immunotherapy in treating apple allergy in a controlled but open trial[61]. Our clinical trial supports earlier claims that birch pollen IT has a beneficial effect on cross-reactive food allergies. Clinical improvement was assessed by DBPCFC for the first time. Of course real proof of clinical efficacy will have to come from a trial in which not only the evaluation, but also the treatment is double-blinded and placebo-controlled. Such a study is now under preparation.

There is some evidence and concern that pollen immunotherapy sometimes induces new IgE specificities[62-64]. Anecdotal data mainly from France suggests that immunotherapy for house dust mite can result in new IgE antibodies that subsequently lead to cross-reactive food allergy, mainly to snails[65,66]. One of the striking observations was that patients experienced inflammatory reactions at the site of injection of mite extract after eating snails. A study by van Ree *et al.* indeed reported induction of IgE during mite IT[67]. Although suggestive, this study was retrospective without placebo controls and we therefore cannot rule out the possibility that the observed "induction" was actually the natural course of the disease. In our study we tried to evaluate possible induction of food allergy (apple) during birch pollen immunotherapy. We did not include these data in the final study because we were not very successful in recruiting birch pollen allergic patients without apple allergy. Of 4 patients without prior sensitization to Mal d 1, two demonstrated a transient positive SPT for Mal d 1. The actual risk of induction of food allergy will certainly still have to be addressed in a double-blind placebo controlled trial on a larger scale.

Immunotherapy with hypoallergenic mutants

Of course the real problem in food allergy is not mild OAS caused by apples but true food allergy like severe peanut allergy leading to a serious decrease in the quality of life of patients and their families. Effective treatment for such patients would really be a major step forward. This has been tried in the past, but severe side effects like anaphylactic shock and even death have prevented further advances[68]. Molecular biology now offers the perspective of production of recombinant mutants with significantly decreased allergenicity. For peanut several hypoallergenic mutants were produced but they have not yet been assessed in vivo[69-71]. The main reason for this is the persisting fear of serious adverse reactions. Decreases in IgE-binding were not always impressive and in some cases a proposed hypoallergen proved to be hyperallergenic for individual patients. To safely evaluate the feasibility of hypoallergens for immunotherapy, we chose Mal d 1 as a model allergen knowing that this allergen never induces severe reactions. By *in vitro* techniques as well as SPT and DBPCFC hypoallergenicity was confirmed in a limited group of patients. The decrease in allergenicity was about 90%. These observations will however have to be extended to a larger population.

An unresolved question is how hypoallergenic 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 depends among other things on the route of administration. At present there are essentially two possibilities: subcutaneous or sublingual application. It is currently thought that effective

treatment by the subcutaneous route requires 100-1000 times lower allergen than sublingual. The required degree of hypoallergenicity will therefore most likely be different for the two routes of administration, also because there are differences in target organ sensitivity.

Another therapeutic approach that has been proposed and evaluated for food allergic patients is targeting of the IgE antibodies with anti-IgE. For a group of peanut allergic patients (mild to moderate peanut allergic patients), administration of anti-IgE antibodies raised the threshold of peanut that they could tolerate[72]. However, despite clinical improvement, this treatment does not induce long term tolerance; its effect is only temporary. Perhaps there is more perspective in using anti-IgE treatment as a pre-treatment before starting allergen-specific immunotherapy with hypoallergens[73]. In that way the risk of anaphylactic side effects could be reduced even further as compared to simply using hypoallergenic mutants. All together these new developments for the first time provide promising perspectives for the treatment of food allergy.

In summary, this study has provided further prove for the great potential of purified major food allergens for the improvement of food allergy diagnostics. The clinical predictive value of detected food allergen-specific IgE antibodies can become much more reliable. In addition, individual and population differences in IgE profiles against individual food allergens provide information that can lead to the identification of the sensitizing allergen source. In addition, geographic differences can be linked to differences in environmental and dietary exposure. Reliable data on the relation between exposure and sensitization are absolutely essential when designing new strategies to prevent and treat food allergy. Our study has provided some promising new leads to improve the treatment of food allergy. For the first time, the effect of birch pollen immunotherapy on cross-reactive food allergy has been monitored by the use of purified major food allergens and by DBPCFC. The results have confirmed earlier claims that cross-reactivity can benefit patients during immunotherapy. Finally, our experiments have shown that biotech foods with decreased allergenicity are feasible. This could decrease the burden of dietary restrictions.

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Summary

It is generally assumed that the incidence of allergies in the Western world has been rising over the past few decades. It is against this background that the European Commission decided to put food allergy on their research agenda. This thesis is part of a multi center collaborative study, the SAFE project, funded by the EU Fifth Framework Program. The acronym SAFE stands for "Plant food allergies: field to table strategies for reducing their incidence in Europe". Apple allergy was chosen as a model system because apples are widely consumed in Europe and they frequently (1-2% of the population in Europe) cause allergic reactions with a variable degree of severity. In Northern and Central Europe, apple allergy has always been described as a mild disease (itching and swelling of lips, mouth and throat). More recent observations from Southern European countries indicate that apple can induce severe systemic reactions as well. The SAFE project aimed at addressing the various aspects of food allergy from sensitization to clinical presentation, as well as at evaluating strategies for prevention and treatment.

Chapter 2 describes the patient population studied. Patients from four countries in Europe were included, specifically from Austria, Italy, Spain and The Netherlands. Different sensitization patterns were studied. Detailed IgE serology was performed using pollen extracts, food extracts and purified apple allergens. The results of these analyses were compared to clinical histories and skin tests. Our studies in four European countries have confirmed that the presence of birch trees is a dominant factor in sensitization to the major apple allergen Mal d 1. Primary sensitization to the major birch pollen allergen Bet v 1 is at the basis of IgE recognition of Mal d 1 in Northern and Central Europe. In Madrid where birch trees are virtually absent the mean IgE response to Mal d 1 was low. The explanation for the absence of Bet v 1-related fruit allergies in Spain is thus straight forward (i.e. the absence of birch trees). However, it is less clear why sensitization to lipid transfer protein (LTP) occurs almost exclusively in Mediterranean countries. Apple allergy in Spain is most likely a result of primary sensitization to peach. Whether the virtual absence of LTP-related food allergy in The Netherlands and Austria is caused by lower consumption levels of fruits like peach is still unclear. It can also not be ruled out completely that specific pollen in the Mediterranean are at the basis of LTP sensitization.

In *Chapters 3 and 4* two novel forms of cross-reactive food allergies among birch pollen allergic patients are presented, i.e. to jackfruit and sharonfruit. In both cases patients reacted on the first exposure to the fruit involved and more severely than they used to do on apple and other fruits. These studies suggest that some food homologs of Bet v 1 might be more stable than the extremely labile Mal d 1 from apple. Jackfruit and sharonfruit allergy can be added to the list of birch pollen related food allergies. Increased consumption of these fruits will result in a rise in allergic symptoms.

Treatment of food allergic patients is primarily based on avoidance diets. Such diets can have a considerable social impact and lead to nutritional deficiencies. *Chapter 5 and 6* describes possibilities to develop hypoallergenic fruit.

In *Chapter 5* the allergenicity of different apple cultivars was assessed by SPT and double-blind, placebo controlled food challenge. Golden Delicious has shown to be a representative of apple cultivars with high allergenicity and Santana consistently demonstrated the lowest

allergenicity. It has also been found that allergenicity increased during storage and that this increase could partly be prevented by reducing oxygen to 2.5% during storage.

Chapter 6 describes the development of a hypoallergenic apple plant in which the Mal d 1 gene was knocked out by RNA interference. Using leaf material of young plantlets for SPT and immunoblot, it was demonstrated that Mal d 1 activity was indeed reduced significantly in the mutant plantlets. Normally it takes around five years to grow trees that produce fruits, so over 5 years we would be able to test using the golden standard the double-blind, placebo controlled food challenge to verify, if the mutant apple is indeed hypoallergenic.

Public opinion on genetically modified foods has proven to be perhaps a bigger hurdle. The appearance of genetically modified foods in the marketplace has resulted in an emotional and fierce public debate and scientific discussion with broad media coverage. The attitude of the allergic patients towards hypoallergenic genetically modified foods was investigated and described in *Chapter 7*. Allergic participants in the study identified a number of benefits to themselves, such as being able to eat the food again to which they are allergic, and being able to eat all food without worries and not to have to carefully check labels.

Finally, two chapters focus on strategies for allergen-specific immunotherapy. For the treatment of inhalant allergies specific immunotherapy was already described in 1900 and is now widely used as an effective treatment of pollinosis, animal, mite and insect venom allergy. In contrast, specific immunotherapy is not used to treat food allergies. A major problem of immunotherapy using food allergens is the risk of severe side effects, including anaphylactic shock.

In *Chapter 8,* a study using birch pollen immunotherapy trial is described in which the effect on cross-reactive apple allergy is evaluated by SPT and DBPCFC. Our clinical trial supports earlier claims that birch pollen immunotherapy has a beneficial effect on cross-reactive food allergies. For the first time the clinical improvement was assessed using double-blind, placebo controlled food challenge.

In *Chapter 9*, a recently developed hypoallergenic mutant of the major apple allergen Mal d 1 is evaluated *in vivo* using SPT and DBPCFC. It was demonstrated that the mutant indeed behaved as a hypoallergen. This was also confirmed by *in vitro* experiments. Hypoallergenic mutants are potential candidates for future use in immunotherapy.

Samenvatting

Algemeen wordt aangenomen dat de incidentie van voedselallergieën in de Westerse wereld de laatste decennia toeneemt. Dit heeft de Europese Unie er toe aangezet voedselallergie op de onderzoeks agenda te zetten. Het onderzoek in dit proefschrift maakt deel uit van een multicenter studie, genaamd SAFE, met Italië, Nederland, Oostenrijk en Spanje als participerende landen en werd gesubsidieerd door de Europese Unie.

Appel allergie is als model voor voedselallergie gekozen omdat appels veel gegeten worden in Europa en omdat appel allergie frequent voorkomt (1-2% in Europa) met een wisselende ernst van de klachten. In Noord en Midden Europa zijn de klachten mild (jeuk in de mond/keel, gevoel van zwelling en benauwdheid), maar in Zuid Europa kan appel allergie resulteren in een anafylactische shock.

Het SAFE project omvat alle aspecten van voedselallergie, van sensibilisatie tot klinische symptomen, als ook strategieën voor preventie en behandeling.

Hoofdstuk 2 beschrijft de patiënten populatie, afkomstig uit de vier participerende landen. De sensibilisatie patronen van de patiënten van Zuid, Noord en Midden Europa werden in dit onderzoek bestudeerd. Dit hebben we gedaan door specifiek IgE te bepalen voor pollen, appel allergenen en andere voedsel allergenen. Resultaten van deze analyses zijn vergeleken met de klinische achtergrond en resultaten van huidpriktesten. De resultaten uit het SAFE project hebben eerdere waarnemingen bevestigd namelijk dat de aanwezigheid van berkenbomen in Noord en Midden Europa een dominante factor is voor de sensibilisatie voor het appel allergeen, Mal d 1. Het Mal d 1 en het berkenallergeen, Bet v 1, zijn homoloog aan elkaar en door de sensibilisatie van het Bet v 1 allergeen wordt men ook gesensibiliseerd voor het Mal d 1 allergeen. Dit fenomeen heet kruisallergie. De symptomen die gepaard gaan met allergie voor Mal d 1 zijn vrijwel altijd mild.

In het bloed van de Spaanse appel allergische patiënten waren nauwelijks IgE antilichamen tegen specifiek IgE voor Mal d 1 detecteerbaar. In de regio rond Madrid komen berkenbomen niet voor en dit is de verklaring dat het Mal d 1 bij de Spaanse appel allergische patiënten zo laag is. Zo duidelijk als de verklaring is voor de afwezigheid van Bet v 1- gerelateerde fruitallergieën in Spanje, zo moeilijk is het om uit te leggen waarom de ernstige symptomen, welke gerelateerd zijn aan sensibilisatie voor het lipid transfer protein (LTP), vrijwel exclusief in Mediterrane landen voorkomen. In Spanje gaat allergie voor appel meestal samen met een sensibilisatie en allergische symptomen voor perzik, en is deze doorgaans ernstiger van aard. Het is nog onduidelijk of de afwezigheid van LTP gerelateerde voedselallergie in Nederland en Oostenrijk wordt veroorzaakt door lagere consumptie van fruit zoals perzik. Een andere verklaring zou kunnen zijn dat de sensibilisatie voor appel in Zuid-Europa via een ander type pollen verloopt, dat LTP bevat, zoals Plataan pollen.

In *hoofdstuk 3 en 4* worden twee exotische fruitsoorten: jackfruit en sharonfruit beschreven, waarvoor berkenpollen-gesensibiliseerde patiënten allergisch kunnen zijn. Voor beide vruchten zagen wij dat patiënten al bij de eerste keer dat ze de vruchten aten, een allergische reactie vertoonden. Dit is een teken dat er sprake is van een kruisallergie. Tevens waren de reacties voor jackfruit/ sharonfruit ernstiger dan na het

eten van appel en andere fruitsoorten. Resultaten in deze hoofdstukken laten zien dat Bet v 1-gesensibiliseerde patiënten op sommige fruitsoorten toch met ernstige symptomen kunnen reageren, terwijl in het algemeen wordt aangenomen dat Bet v 1 verwante allergenen in fruit, slechts milde allergische symptomen veroorzaken.

Patiënten met een voedselallergie wordt geadviseerd het betreffende allergeen waarvoor men allergisch is te vermijden. Aangezien patiënten meestal niet alleen allergisch zijn voor appel, maar voor meerdere *Rosaceae* fruitsoorten zoals bijvoorbeeld: peer, perzik, pruim en kers, bestaat de kans op het ontwikkelen van vitamine gebrek.

Een alternatief voor allergeen vermijding is om hypoallergeen voedsel te ontwikkelen. *Hoofdstukken 5 en 6* geven hiertoe een eerste aanzet. In *hoofdstuk 5* werd de allergeniciteit van verschillende appelsoorten onderzocht door middel van huidpriktesten en dubbel-blind placebo-gecontroleerde provocaties. Uit onderzoek bleek dat Golden Delicious één van de meest allergene appels is en dat Santana slechts beperkte allergeniciteit bezit. Er werd ook gevonden dat allergeniciteit toeneemt gedurende de opslag van appels, en deze toename gedeeltelijk kan worden voorkomen door het zuurstofgehalte op 2.5% te houden gedurende de opslag. Deze waarnemingen kunnen in de toekomst wellicht bijdragen tot het ontwikkelen van hypoallergene appels.

Hoofdstuk 6 beschrijft de ontwikkeling van een hypoallergene appel plant, waarin de productie van Mal d 1 wordt geremd door middel van RNA-interferentie. Met behulp van huidpriktesten en immunoblot werd vastgesteld dat jong bladmateriaal van de gemuteerde appel plant minder Mal d 1 bevat. De meest optimale test om aan te tonen dat gemuteerde appels verkregen langs deze weg, minder allergeen zijn, is een dubbel-blind placebo-gecontroleerde provocatie met deze appels. Dit kan echter pas over ongeveer vijf jaar getest worden als er appels aan de gemuteerde appelboom hangen.

De introductie van genetisch gemodificeerd voedsel op de markt heeft geleid tot zowel veel emotionele als wetenschappelijke discussies. In *hoofdstuk 7* onderzochten we de houding van voedsel-allergische patiënten ten opzichte van genetisch gemodificeerd hypoallergeen voedsel. Voedsel-allergische patiënten in deze studie zagen duidelijk voordelen van dergelijke producten. Enkele genoemde voordelen zijn: het weer in staat zijn om voedsel te eten waarvoor zij allergisch zijn, zonder angst voor een allergische reactie, en niet meer gedwongen zijn om altijd zorgvuldig labels op voedingsproducten te hoeven lezen.

De laatste twee hoofdstukken zijn toegespitst op immunotherapie. Immunotherapie is een behandeling waarbij de patient gedesensibiliseerd wordt met veelvuldige injecties voor het allergeen waarvoor men allergisch is. Inhalatie-allergie wordt al sinds jaren met succes behandeld met allergeen-specifieke immunotherapie. In *hoofdstuk 8* is onderzocht of de behandeling van berkenpollen allergie ook een gunstig effect heeft op een kruisreactieve voedsel allergie. Deze studie laat voor het eerst met behulp van dubbel-blind placebo-gecontroleerde provocaties zien dat berkenpollen immunotherapie inderdaad ook werkzaam is voor de aan berkenpollen gerelateerde appel allergie.

Het behandelen van ernstige niet pollen-gerelateerde voedselallergie met behulp van immunotherapie wordt niet toegepast, omdat dit aanleiding geeft tot ernstige bijwerkingen, zoals anafylactisch shock. Het is dus noodzakelijk een veiliger manier van immunotherapie voor de behandeling van voedsel allergie te vinden. Een mogelijke oplossing voor een veilige immunotherapie met voedsel allergenen is het muteren van de voedselallergenen tot hypoallergene varianten. Om te beoordelen of deze benadering

klinisch haalbaar is, beschrijft *hoofdstuk 9* of een gemuteerd Mal d 1 molecuul *in vivo* hypoallergeniciteit vertoonde. Dit werd geëvalueerd met behulp van huidpriktesten en dubbel-blind placebo-gecontroleerde provocaties. Het gemuteerde allergeen bleek inderdaad minder allergeen dan het wildtype molecuul. Dit werd tevens ondersteund door *in vitro* experimenten. Immunotherapie met dergelijke hypoallergene mutanten kan in de toekomst een mogelijke behandeling zijn van voedselallergie.

Dankwoord

C'est fini! Met zeer veel plezier heb ik de afgelopen jaren gewerkt aan dit promotie-onderzoek, maar ben ook weer blij dat het nu bijna is afgerond. Eindelijk kan ik eens iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift.

Allereerst wil ik alle patiënten bedanken die zich beschikbaar hebben gesteld voor het onderzoek met daarbij het doorstaan van de vele huidpriktesten, bloed afnamen en provocaties. Zonder u was dit onderzoek zeker niet tot stand gekomen. Hartelijk dank hiervoor!

Het geluk is dat ik gesteund werd door twee co-promotoren.

Dr. Ronald van Ree, graag wil ik je bedanken voor je tomeloze inzet. Je was voor mij de Engel van het onderzoek en altijd bereid om te helpen. Ik hoop dat je al je kwaliteiten op korte termijn verder tot stand kunt brengen hier op de afdeling Dermatologie van het UMC Utrecht.

Dr. André Knulst, graag wil ik je bedanken voor het grote enthousiasme voor de voedselallergie die je me de afgelopen jaren hebt weten over te brengen. Bedankt voor de vrijheid en het vertrouwen die je me gaf.

Prof. Dr. Carla Bruinzeel-Koomen, mijn promotor, bedankt voor het in staat stellen van het mogelijk maken van dit promotie onderzoek en de soepele overgang naar de opleiding. Bedankt voor je interesse in mijn onderzoek en het doorlezen van alle manuscripten zelfs in de vroege uurtjes.

Zonder Laurian en Astrid zouden er heel wat lege bladzijden zijn ontstaan in dit proefschrift. Bedankt voor het doen van alle proeven. Laurian, jouw inzet en interesse was enorm en zelfs tijdens je zwangerschapsverlof heb je nog heel wat dingen voor mij gedaan. Natuurlijk wil ik kleine Luka ook bedanken dat hij net na zijn geboorte samen met jou nog plaatjes heeft ge-layout, want het moest af. Bedankt!!

Luud Gilissen en Eric van de Weg, bedankt voor de samenwerking, uiteindelijk hebben we er toch nog 2 mooie artikelen uit kunnen persen. Bedankt hiervoor.

I would like to thank all participants of the SAFE group and especially Karin Hoffmann-Sommergruber, the manager of the SAFE project, for their cooperation and for the pleasant atmosphere in which we have had our meetings in the various countries.

Mijn kamergenoten: Kim, niet alleen kamergenoot en appel allergisch onderzoeks patiënt, maar ook een hele gezellige buurvrouw en skimaatje, bedankt voor alle gezelligheid tijdens en buiten het werk en voor als het echt nodig was de Willeke Alberti momenten. Annebeth altijd in voor gekkigheid, succes met het verdere onderzoek. Machteld, bedankt voor de goede en gezellige samenwerking, dat hoofdstuk immunotherapie hebben we toch maar mooi gedaan. Bert, onze enige man op de kamer, af en toe werd je gek van de Willeke

momenten maar je hebt ze toch maar goed doorstaan. Els, voor alle immunologische vragen kon ik altijd bij je terecht, bedankt hiervoor.

Natuurlijk wil ik onze energievolle en enthousiaste hoofd onderzoeker Edward Knol bedanken. En daarbij ook de andere onderzoekers: Dirk Jan, Chantal, Evert, Inge, Marloes, Annemiek, Adri, Mayke K, Mayke H en Ilze bedanken voor de gezelligheid tijdens de koffiepauze met soms aansluitend de lunch en de borrels. De secretaresses Marian en Jantine horen hier natuurlijk ook bij. Marian jammer dat je weg bent, de gesprekken, discussies met jou en Chantal waren altijd zeer boeiend. Jantine altijd bereid om weer voor de zoveelste keer, ook al ben je appelallergisch een appel te eten en huidpriktesten te doorstaan. Aan bijna ieder hoofdstuk van dit proefschrift heb jij wel deelgenomen.

Andrea, bedankt dat je veel stukken op het Engels gecorrigeerd hebt, ook al zit je nu niet meer bij ons op de afdeling, maar ver weg in Arizona.

Corinne, bedankt voor de helpende hand bij de provocaties, nu nog in november de eindsprint.

De dames van de allergie, bedankt voor jullie gastvrijheid en dat jullie toch nog altijd een kamer voor mij vrij hadden, als ik weer eens op een onaangemeld tijdstip kwam aanvliegen.

De huidige arts assistenten pool, bedankt voor de soepele opstelling van mijn eerste stages van mijn opleiding.

Marja, mijn 50% SOA maatje, wat hebben wij gelachen! Dat onderzoekje moeten we toch eens verder uitwerken. Succes met de laatste loodjes van je onderzoek.

Ines, bedankt voor de gezelligheid en dat je de eerste twee weken op zaal hebt overgenomen en dat je me alle ins and outs van zaal hebt gewezen. Natuurlijk wil ik al die biopten, die op onmogelijke tijden moeten worden afgenomen, voor je afnemen.

Dan mijn golf, fitness en skimaatjes: bedankt voor alle sportieve uitjes, ontspanning, gezelligheid en etentjes. Met name Rebecca die op al deze 3 fronten er altijd bij is en een super organisator is. Rebec bedankt voor alle leuke, gekke en toch ook wel soms serieuze gesprekken die wij tijdens golf, fitness en skiën hebben.

Mijn “geneeskunde” vriendinnen bedankt! Er zijn er een paar die al jaren zitten te zeuren dat als er dan echt uiteindelijk een boekje komt, ze graag met naam en toenaam genoemd willen worden. Nou daar komen jullie dan. Marianne samen begonnen we na de co-schappen in het UMC. Helaas zit je nu niet meer in UMC, maar met alle bakjes koffie die we op C2 hebben gedronken, hebben we zeker voor een paar jaar reserves gecreëerd. Marijke en Marieke, jullie zijn nog de enige die in het UMC zijn gebleven, het “intercollegiale” overleg in en buiten het UMC waardeer ik dan ook zeer. Zita en Nayyirih, allebei zijn jullie onderzoek en oogheelkunde gaan doen. Bedankt voor het uitwisselen van de promotieperikelen en succes met het afronden van jullie promotie.

Mijn paranimf, Caroline. Mijn lieve grote zus Caroline ging naar Utrecht om geneeskunde te studeren, twee jaar later ik er achteraan. Vanaf het moment dat ik naar Utrecht kwam heb ik

minstens 1 x per week een maaltijd bij je genuttigd. Nu wordt het tijd dat de rollen worden omgedraaid. Lieve Caroline, bedankt dat je mijn paranimf wil zijn.

Mijn paranimf, mijn lieve goede vriendin Monique, vanaf het begin van de studie liepen onze wegen parallel, zelfde stages en samen naar Malawi, het grote avontuur. Jij nu de huisartsen opleiding en ik de dermatologie opleiding en ook hier zijn weer veel gelijkenissen. Bedankt dat je mijn paranimf wil zijn.

Dan wil ik mijn lieve gezellige familie bedanken, die de laatste jaren explosief groter is geworden met alle kleintjes die er zijn en de kleine die op komst is. Lieve Christianne, Charlotte, Loek jr, Fréderique en Thijs bedankt voor de vooral leuke en ontspannende momenten.

Mijn grote lieve zus Lucienne, als ik geen zin heb om te koken is Lucienne een van de adressen waar ik mezelf graag bij uitnodig. Lucienne bedankt dat je altijd met raad en daad voor me klaar staat.

Dan natuurlijk mijn zwagers: Arjan en Jalmar, bedankt dat jullie het toch altijd maar weer accepteren als jullie schoonzusje weer eens voor de zoveelste keer bij jullie aan tafel schuift. Bedankt voor jullie gezelligheid.

Natuurlijk mag ik tante Trees niet vergeten te bedanken. Door jou werd ik er altijd aan herinnerd of ik wel geheel op schema zat met mijn onderzoek, dit natuurlijk op aansporen van oom Theo. Bedankt voor jullie interesses.

Lieve Loek en Marijke, bedankt voor jullie uitnodigingen zodat ik mijn energie kwijt kon om te komen klussen in jullie “vakantiehuisje” en tuin. Ik verwacht dat er nog vele zullen volgen, deze zal ik dan weer met veel plezier aanvaarden.

Lieve papa en mama, jullie hebben mij altijd gestimuleerd om eruit te halen wat erin zit en staan daarmee aan de basis van dit proefschrift. Dank jullie wel dat jullie nog steeds altijd voor mij klaar staan.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 12 maart 1974 te Venlo. Na het behalen van het Atheneum-B diploma aan het Collegium Marianum te Venlo, begon zij in 1993 aan de studie geneeskunde aan de Universiteit van Utrecht en behaalde in januari 2000 haar artsexamen. Tijdens haar studie was zij werkzaam als beheerder van het vaardighedenlaboratorium van de opleiding huisartsgeneeskunde aan de Universiteit van Utrecht. In 1997 in de wachttijd van haar co-schappen heeft ze 4 maanden lang onderzoek verricht naar de oorzaak van geruptureerde uterus in het Queens Hospital, te Liwonde, in Malawi. Na terugkeer is ze gestart met een onderzoek naar arbeidsdermatose op de afdeling Dermatologie en Allergologie van het Universitair Medisch Centrum Utrecht.
Na het behalen van haar artsexamen is ze in februari 2000 gestart als arts-onderzoeker op de afdeling Dermatologie en Allergologie van het Universitair Medisch Centrum Utrecht. In eerste instantie verrichtte ze diverse trials naar het effect van Tacrolimus bij constitutioneel eczeem bij kinderen. In 2001 is zij begonnen met het SAFE project dat uiteindelijk tot dit proefschrift leidde. Op 1 oktober 2003 is zij begonnen met de opleiding Dermatologie in het Universitair Medisch Centrum Utrecht.

Abbrevations

A	Austria
Ara h	Arachis hypogea
Bet v	Betula verrucosa
BHR	Basophil histamine release
BPE	birchpollen extract
BSA	Bovine serum albumine
CCD	crossreactive carbohydrate determinants
CU	contact urticaria
DBPCFC	double-blind, placebo controlled food challenge
DIECA	diethyldithiocarbamate
E	Spain
EAST	enzyme allergosorbent test
EDTA	ethylenediaminetetraacetic
ELISA	enzyme-linked immunosorbent-assay
FEIA	fluorescent enzyme immuno assay
Gly m	Glycine max
I	Italy
IgE	immunoglobuline E
IT	immunotherapy
LTP	lipid transfer protein (Mal d 3)
Mal d	Malus domestica
NL	the Netherlands
OAS	oral allergy syndrome
OC	open challenge
Par j	Parietaria judaica
PBS	phosphate buffered saline
PCBER	phenylcoumaran benzylic ether reductase
PR	pathogenesis related
PVPP	polyvinylpolypyrrolidone
r	recombinant
RAST	radio allergosorbent test
RC	rhinoconjunctivitis
RI	RAST-inhibition
RNAi	RNA interference
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFE	sharonfruit extract
SPT	skin prick test
PTGS	post-transcriptional silencing of specific genes
TLP	thaumatin-like-proteins (Mal d 2)
Tr1	type 1 regulatory T cells
Tregs	regulatory T cells
UMCU	University Medical Center Utrecht
VAS	visual analogue scale
WT	wild type