Refined tools to define hazelnut and peanut allergy in children

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Verfijnde methoden om
hazelnoot en pinda allergie
bij kinderen te definiëren

(met een samenvatting in het Nederlands)

Proefschrift

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General Introduction

Peanut and hazelnut sensitization and allergy in childhood

Peanut (Arachis hypogea) and hazelnut (Corylus avellana) are notorious for causing allergic reactions in childhood. [1] After exposure of allergic children to the culprit peanut or hazelnut, the first symptoms of an allergic reaction usually occur within one hour. [2] Symptoms can occur in several organ systems, such as the oral cavity (itching, swelling of the mouth and/or throat), the skin (urticaria, angio-edema), the gastro-intestinal tract (abdominal pain, vomiting, diarrhea), the airways (rhinoconjunctivitis, laryngeal edema, bronchoconstriction) or the cardiovascular system (tachycardia, hypotension, shock). [3] Fatal and near-fatal reactions include (ir)reversible bronchoconstriction and anaphylactic shock and occur in approximately 8-10 subjects per 100,000 inhabitants. [4;5] Peanut and tree nuts (like hazelnut) were the most common causes of all fatal and near-fatal allergic reactions to food documented in the US. [6;7]

The annual incidence of an allergic reaction after accidental exposure is estimated at 14-24% for peanut and 7% for tree nuts, including hazelnut. [8;9] Accidental ingestions, caused by sharing food, hidden ingredients or cross-contamination, often occur at home or in school, but also in restaurants. [8;10] To prevent such inadvertent allergic reactions, strict elimination of peanut and/or hazelnut is generally advised. However, dietary restrictions can result in impairment of physical and psychosocial development, because the affected children are isolated and overprotected in order to prevent the ingestion of hazardous food. [8;11;12] Living with food allergy affects the quality of life of both children and their parents. [13-16] Anxiety of the parents about potentially fatal allergic reactions may contribute to this observation.

Few studies have investigated the prevalence of hazelnut or peanut sensitization and allergy. The most recent figures are derived from the UK, with the highest prevalence ever observed of 2.8% for peanut sensitization and 1.8% for peanut allergy in children aged 4-5 years. [17] Earlier studies in 2 sequential cohorts born in 1989 and 1994 on the Isle of Wight reported an increase in sensitization to peanut from 1.1% to 3.3% and in allergy from 0.5 to 1.0% at 4-6 years of age. [18] In the US, the estimated prevalence of peanut and/or tree nut allergy is 1.1% based on a telephone survey. [19] Cross-sectional studies showed that the prevalence of hazelnut allergy ranges from 0.4-0.7% in westernized countries. [20;21] Once peanut allergy has been established, few children will outgrow this condition. [22-24] Moreover, in 8% of the children with resolution of peanut allergy, symptoms can recur. [25] No figures about resolution of hazelnut allergy exist. Taken together, approximately 1% of children suffer from peanut and/or hazelnut allergy, with a risk for serious allergic reactions that can persist throughout life.

Need for an adequate diagnosis and the role of double blind placebo controlled challenge (DBPCFC)

Peanut and hazelnut sensitization in children must be taken seriously, but the prevalence of sensitization (around 3% for peanut in the UK) exceeds the prevalence of allergy (1-2%). [17;18] This suggests that 30-70% of sensitization is not clinically relevant. Current practice is to screen for sensitization to food allergens mainly in children with atopic dermatitis, because 30% of atopic dermatitis in childhood coincides with food allergy. [26;27] Together with determination of allergen-specific IgE, an accurate clinical history should be obtained about ingestion of peanut or hazelnut and possible allergic reactions. If a child eats peanut without occurrence of allergic symptoms, sensitization is not clinically relevant. On the other hand, if a child reports a previous allergic reaction after the ingestion of peanut or hazelnut, the child has a significant chance to be allergic. The diagnosis becomes difficult when the child is sensitized without a history of previous exposure to peanut or hazelnut; in the Netherlands, and also in the UK and in the US, the ingestion of peanut and hazelnut is advocated to be postponed until after the age of 3. [28;29] Another factor complicating the history is that peanut and hazelnut are notorious as hidden ingredients in any number of food products, and therefore accidental ingestion may not always be acknowledged. [30;31] Children find it particularly hard to distinguish the peanut or tree nut to which they are allergic. [32] In conclusion, if the history is negative or does not fit with the pattern of sensitization there is a need for oral challenge. The best and safest way is to perform this is a double blind placebo controlled food challenge (DBPCFC) in the hospital. [33] During DBPCFC, the suspected food is administered in gradually increasing the doses, alternated with placebos. The individual eliciting dose to develop symptoms can hence be determined. [34] Once allergic symptoms occur, the challenge is stopped and appropriate medication is supplied. Children who do not develop allergic reactions may introduce peanut or hazelnut in their diets. Children who develop allergic reactions are advised to eliminate the culprit food from their diet.

Strict elimination is especially mandatory in allergic subjects who may react to tiny amounts of contamination in food products. Previous studies in adult patients showed that individual thresholds can vary enormously, with doses eliciting allergic symptoms as low as 100 µg peanut or 1 mg hazelnut protein, reflecting 1/1500 peanut or 1/200 hazelnut. [35-37] Such data for children are lacking because allergic reactions were reported after the first dose. [38-40] So far, the lowest reported eliciting dose in children is 2.5 mg peanut protein. [38]

Taken together, DBPCFC is an important tool to confirm the diagnosis of peanut and hazelnut

allergy in sensitized children; furthermore, it allows determination of the threshold dose that elicits allergic symptoms. [34]

Determination of sensitization to major allergens as a method to define the clinical reactivity to peanut and hazelnut: component resolved diagnosis

Although DBPCFC is regarded as the gold standard to determine the clinical relevance of sensitization to peanut or hazelnut, this method is expensive and time-consuming. Furthermore, DBPCFC can potentially elicit serious allergic reactions. [41] Therefore, it would be worthwhile to develop non-invasive diagnostic methods that can replace the DBPCFC in the future.

Although plant foods, to which peanut and hazelnut belong, contain numerous amounts of different proteins, only some of these proteins have been implicated as allergens. [42] Major plant food allergens can be divided in 3 groups. [43] The prolamin superfamily comprises 2S albumins, such as Ara h2 and Ara h6 in peanut, and lipid transfer proteins (LTP), such as Cor a 8 in hazelnut. [44;45] Members of another superfamily, the cupins, include 7S globulins, such as vicilins Ara h1 in peanut and Cor a 11 in hazelnut, and 11S globulins, of which the legumins Ara h3 in peanut and Cor a 9 in hazelnut are examples. [46-50] The last family consists of cross-reactive homologues of the major birch pollen allergen Bet v 1, to which Cor a 1 in hazelnut belongs. [51]

In children with presumed peanut allergy, IgE specific for Ara h1, Ara h2 and Ara h3 has been reported [52-56]. Peanut-allergic adults recognized Ara h6 to a similar extent as Ara h2, but this finding has not yet been confirmed in children with peanut allergy. [44;57] In birch endemic areas, sensitization to hazelnut is mostly ascribed to the Bet v 1 homologues in hazelnut, such as Cor a 1. [58;59]

The clinical reactivity of patients to peanut and hazelnut is partially dependent on the structure of the allergens. [60] Proteins that keep their structure after heating and digestion are able to reach the intestinal tract intact. [61] The 2S albumins, for instance Ara h2 and Ara h6 in peanut, and lipid transfer proteins (LTP), like Cor a 8 in hazelnut, are examples of heat and digestion resistant proteins and are related to more serious allergic reactions. [62] In contrast, Bet v 1 homologues are rather unstable to heat and digestion, usually causing symptoms restricted to the oral cavity. [58;59] Exposure to peanut and/or hazelnut may give rise to differentially severe symptoms depending on which allergen component(s) the individual is sensitized to. Analysis of the allergen sensitization pattern through use of purified natural or recombinant allergen molecules rather than crude natural extracts may serve to enhance the predictive and prognostic power of IgE antibody-based

allergy diagnostics [63-65]. This concept has been defined as 'component-resolved diagnostics and may replace the DBPCFC in the future.

Investigation and modulation of T and B cell responses to peanut

The production of IgE by B cells, leading to sensitization, is the result of a complex interaction between the allergens (peanut, hazelnut) and the immune system. After exposure, the food proteins cross the epithelial barrier and contact the immune system. Protein fragments are processed by antigen-presenting cells (APCs) and displayed on their surface in association with major histocompatibility complex (MHC) class II molecules and can be recognized by a specific T cell receptor. In the presence of interleukin (IL)-4, naïve antigen-specific T helper (Th) cells differentiate into effector Th2 cells. Typically, Th2 cells produce a cocktail of cytokines including IL-4, IL-5 and IL-13 that, among other actions, encourage B cells to develop into allergen-specific IgE-producing plasma cells.

Understanding of the immunological mechanisms concerning T cell responses towards peanut remains limited. A recent study reported a dominant Th2 response both in peanut-allergic and sensitized but tolerant adults. [66] In children, one study showed a predominant Th2 response to peanut in allergic children, but Th1 skewing in tolerant children. [67] This is in line with previous studies from our group that showed that specific T cells from children with cow's milk allergy tend to produce Th2 cytokines, whereas cow's milk tolerant children produce more Th1 cytokines. [68] Cow's milk tolerant atopic children had significantly more IL-10 producing T cells. [69] The production of IL-10 in cow's milk tolerant atopic children is also related to higher production of IgG4 by B cells. [70] The precise role of IgG4 is not clear, but it has been postulated that IgG4 may interact with the allergen and thereby prevent IgE binding. [71] Nowadays it is possible to screen for individual IgE and IgG4 binding epitopes in the major peanut allergens simultaneously with minimal invasiveness using peptide micro immunoarrays (MIA). [72] Information obtained from this assay, and also from the T cell responses to peanut allergens, may be used to understand differences in the clinical reactivity to peanut as determined by DBPCFC.

At the present time there is no curative therapy available once peanut allergy has been established. Several approaches can be postulated to modulate the immunological response towards tolerance. The first approach focuses on allergen-specific induction of tolerance, in line with allergen-specific immunotherapy commonly used to treat patients with inhalant allergies. This therapy in peanut-allergic subjects, using native peanut allergens, led to an unacceptably high rate of allergic reactions.

[73] A safer approach may be the use of short peanut peptides for immunotherapy. [74] The advantage of using peptides for immunotherapy is that peptides can stimulate the T cells while avoiding the risk of anaphylactic responses because the peptides lack the ability to crosslink IgE.

A second approach could be based on the modulation of the Th2 response in general. The 'Hygiene' hypothesis explains the increased prevalence of allergy by a diminished exposure to microbes in the ecosystem, leading to a suboptimal balance between Th1 and Th2 immunity. Probiotics are live microorganisms, such as lactic acid bacteria, that confer a health benefit on the host when administered in adequate amounts. In vitro, probiotics were shown to have a strong potency to induce Th1 and regulatory responses. [75-77] According to these in vitro studies, probiotics may be beneficial in atopic subjects. [78;79] However, the effect on allergen-specific immune responses has not been studied before now. We investigated whether in vitro immunological effects of probiotics are reflected in effects on allergen-specific responses after oral treatment with probiotics.

Aims and outline of this study

Peanut and hazelnut allergy in childhood is associated with a constant risk of potentially fatal reactions. Parents may therefore live with constant fear and vigilance, affecting everyday life. The level of anxiety about such allergic reactions that is associated with peanut and hazelnut allergy was investigated in chapter 2. Currently, a diagnosis of hazelnut and peanut allergy based on sensitization alone is not sufficient. Therefore, the sensitivity of the child should be investigated by DBPCFC, determining the allergic reaction and the eliciting dose of allergic symptoms (chapter 3 and chapter 5). In order to improve current diagnostic tests that may replace DBPCFC in the future, sensitization to different major allergens in hazelnut and peanut, rather than to crude extract, was related to the clinical reactivity of the sensitized child in chapter 4 and chapter 6, respectively. Moreover, the diversity and location of the IgE and IgG4 binding epitopes on each major peanut allergen may be correlated to severity of clinical symptoms, which was studied in chapter 7. In chapter 8, the T cell responses to peanut allergens and peanut peptides in peanut allergic subjects and also in tolerant subjects were investigated. The ability to change the T cell response to peanut after oral administration of probiotics in sensitized subjects was examined in chapter 9.

Finally, the implications of these findings together are discussed in chapter 10.

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Parental anxiety before and after food challenges
in children with suspected peanut and hazelnut allergy

Abstract

Background: As ingestion of peanut and hazelnut by allergic children is potentially life threatening, parents of these children need to be vigilant about their child's dietary intake. This may cause high levels of anxiety. We assessed parental anxiety about a food allergic reaction in their child (state anxiety) and their personal disposition to anxiety (trait anxiety). Parental anxiety was investigated again after food challenges.

Methods: Fifty-seven children (3-16 years, mean age 7.2) with suspected peanut or hazelnut allergy (mean specific IgE 20.9) were evaluated by double-blind, placebo-controlled food challenge (DBPCFC). Thirty-two children (56%) developed an allergic reaction, ranging from mild to severe. The parents of all children completed the Spielberger State-Trait Anxiety Inventory (STAI) prior to DBPCFC and two weeks, three months and one year thereafter.

Results: Prior to DBPCFC, parents had high levels of state anxiety in contrast to a lower trait anxiety than the norm group. A previous inadvertant reaction was significantly related to state anxiety ($B=8.856$, $P=0.021$). After DBPCFC, the state anxiety was significant lower, regardless of a positive or negative outcome ($P<0.05$). The state anxiety was still significant lower after one year ($P<0.03$). The trait anxiety remained unchanged. State and trait anxiety were related at different moments in the study ($P<0.003$).

Conclusions: Parents of children with suspected peanut or hazelnut allergy show high levels of state anxiety about a food allergic reaction in their child. After DBPCFC this parental state anxiety was significantly lower while trait anxiety remained stable troughout the study.

Introduction

Allergic reactions to peanut and hazelnut are associated with potentially fatal outcome.[1] Parents of children with food allergy need to be vigilant about their child's dietary intake, which may cause high levels of anxiety. Anxiety has a major impact on wellbeing. Living with food allergy affects the quality of life of both parents and children.[2-6] Although anxiety could be interpreted as protective, since it increases compliance,[5] living with frequent anxiety about a food allergic reaction is expected to be counterproductive. Anxiety can lead to unrealistic or unfounded restrictions. Parents of teens with food allergy listed fear of death as the most difficult issue.[7] Parental anxiety and expressed fearfulness have been shown to be related to child anxiety.[8] Childhood anxiety can cause considerable distress and impairment. Children reported that their fears interfered with daily activities and prevented them from doing things they like.[9] As far as we know, specific feelings of anxiety about a food allergic reaction have never been reported.

The goal of the present study was to assess (1) parental anxiety about food allergic reactions in their child and (2) the association between a double blind placebo-controlled food challenge (DBPCFC) and parental anxiety about food allergic reactions in their child.

Two important perspectives of anxiety can be distinguished: state anxiety and trait anxiety.[10] State anxiety is a temporary emotional state. It provides a valid indication of change in intensity of transitory anxiety in response to real-life stress, for example anxiety prior to an exam. Trait anxiety is the dispositional or general anxiety that is expected to be rather stable over time. We distinguished anxiety about a food allergic reaction (state anxiety) and the personal disposition to anxiety in parents (trait anxiety). In the present study population, children sensitized to peanut and hazelnut were carefully evaluated by a standardized questionnaire, determination of sensitization, and DBPCFC.[11] Our research questions were (1) are suspected peanut and hazelnut allergies in children associated with high levels of parental anxiety about allergic reactions of their child, and (2) will these levels of anxiety be lower, especially when the diagnosis can be refuted, and remain lower one year after DBPCFC. Furthermore, we expected that the trait anxiety of parents would be constant.

Methods

Study group

The study group (n=57), further referred to as the challenge group, consisted of the parents of 37

boys and 20 girls (3-16 years) who underwent a DBPCFC for peanut (n=33) or hazelnut (n=24) in the Centre of Pediatric Allergology. Both groups did not differ in gender, age, parental educational level and atopic activity (data not shown). The mean specific IgE of the study group was 20.9 and 40% had an unexpected food allergic reaction prior to the study (table I). The children participated in a study to determine no-observed adverse-effect levels and eliciting doses for peanut and hazelnut.[11;12] All challenges were supervised by one medical doctor in a clinical setting equipped for resuscitation and monitoring of vital signs. According to the DBPCFC, 22 children had peanut allergy (67%) and 10 had hazelnut allergy (42%). Reactions varied from mild oral allergy (n=5) to more serious reactions including symptoms of the airways and cardiovascular system (n=6). In total, the diagnosis of peanut or hazelnut allergy could be refuted in 25 children (44%). Because 53% of the children were younger than seven years and the differences in ages, we only asked the parents about anxiety about a food allergic reaction in stead of the children and parents both.
All parents gave written, informed consent. The study was reviewed and approved by the Central Committee of Human based Research in The Netherlands (CCMO, The Hague, The Netherlands).

Refusal group

A group of sixty parents of children with peanut allergy who refused to participate in the DBPCFC or who did not respond to the first invitation were approached (further referred to as refusal group). Thirty-three children of this group (55%) had a previous food allergic reaction.
Most important reasons not to take part in the DPBCFC were that the parents thought the DBPCFC was too demanding for the child (25%) or the parents were anxious about the reaction (13%). The refusal group was asked to complete the anxiety scale once.

Psychological Measures

We used the Spielburger State-Trait Anxiety Inventory, Dutch Version,[10] to assess two types of anxiety: state anxiety (transient emotional condition, in this study anxiety about a food allergic reaction) and trait anxiety (disposition indicating anxiety proneness). The state and trait subscales both comprise 20 items and can be judged on a 4-point Likert scale. Examples of items of both scales are "I feel stressed" or "I feel undecided". The range of the scale is 20 (low anxiety) to 80 points (high anxiety).
To assess the anxiety of parents about an allergic reaction of their child to peanut or hazelnut, the instruction at the state subscale was: "...imagine your child is offered (food that contains) peanut or hazelnut, how do you feel?...".

Table I. Characteristics of the study group (n=57).

	Total (n=57)
Patient demographics	
mean age (years)	7.2
boys	37 (65%)
Atopic diseases	
atopic dermatitis	47 (83%)
allergic asthma	28 (49%)
seasonal rhinoconjunctivitis	27 (19%)
other food allergies	52 (91%)
Peanut or hazelnut allergy	
mean specific IgE (kU/L)	20.9
previous allergic reaction	23 (40%)*
positive DBPCFC	32 (56%)
Education mother†	
lower training or lower secondary school	14 (25%)
general education or higher secondary school	16 (28%)
higher education or university	20 (35%)
missing	7 (12%)

*Significant difference between peanut and hazelnut group (55% versus 21%, p=0.010)

The STAI is a frequently used questionnaire in research of behavioral medicine. Different studies and general norm groups have been described. Mean state anxiety scores of these groups are: norm group 38.0, women before IVF treatment 44.1, test anxiety 45.5 and pregnant woman after having lost a child to coth death 41.2.[13] The mean trait anxiety score of the norm group is 39.0.[10] The general associations between state and trait anxiety vary from 0.60 to 0.84.[10]

These questionnaires were given to the parents at four points during the study: prior to, and two weeks, three months and one year after the food challenge. After one year the state scale to the mothers of children with peanut allergy was given alone as a change of trait anxiety was not to be expected and we did not want to burden the parents with an extra questionnaire.

Statistics

Statistical analysis was done with the SPSS (Statistical Package for Social Sciences) for Windows. Descriptive statistics were used to describe anxiety variables, and the mean anxiety scores were compared with the Dutch norm scores by 1-sample t-tests. Parental anxiety scores measured at different moments were compared using a paired sample t-test. In the analyses that compared groups for state anxiety we investigated the relation between state anxiety and other factors using using linear regression analysis.

Results

Response rates to questionnaires

The state and trait anxiety were investigated in parents of 57 children (33 peanut allergy and 24 hazelnut allergy) who were tested with the DBPCFC. Questionnaires were sent prior to, and two weeks, three months and one year after the food challenge. Prior to the food challenge, 55 mothers (96%) and 49 fathers (86%) completed the anxiety questionnaire. Two weeks after the food challenge, the anxiety questionnaire was completed by 37 mothers (65%) and 29 fathers (51%). After three months, 53 questionnaires were received from the mothers (93%) and 44 from the fathers (77%). After 1 year, 30 questionnaires were returned by the mothers (91%; peanut allergy). In the refusal group, which was not challenged, 31 mothers (52%) and 14 fathers (23%) returned the questionnaires.

Anxiety prior to the DBPCFC

Both the state and trait anxiety were analyzed prior to the DBPCFC. No differences in state and trait anxiety scores were found between the mothers of children with peanut allergy and mothers of children with hazelnut allergy (mean state anxiety peanut group 50.5, SD 13.4, hazelnut group 44.3, SD 14.8, P=0.114; mean trait anxiety peanut group 34.6, SD 6.7, hazelnut group 34.0, SD 7.5, P=0.750). Also no differences in demographic factors were found. Therefore, in the following analyses the mothers of children with peanut allergy and hazelnut allergy were regarded as one study group. Prior to the challenge mothers had high levels of state anxiety compared to the norm group (mean

Table II. Comparisons of state anxiety in mothers before and after DBPCFC

DBPCFC	Negative reaction DBPCFC			Positive reaction DBPCFC		
	n	State anxiety	SD	n	State anxiety	SD
Prior to	24	44.1	14.8	30	51.2	12.8
2 weeks after	18	33.7	9.9	18	48.1	12.7
3 months after	24	34.2	11.3	29	40.3	12.6
1 year after	10	31.2	10.2	20	39.3	14.7
Significant decreases		I II III			I II III IV	

I Significant decrease of state anxiety before and two weeks after DBPCFC, $P \leq 0.01$
II Significant decrease of state anxiety before and three months after DBPCFC, $P \leq 0.01$
III Significant decrease of state anxiety before and one year after DBPCFC, $P \leq 0.01$
IV Significant decrease of state anxiety two months and three months after DBPCFC, $P \leq 0.01$

state anxiety study group 47.9, SD 14.2, norm group 38.0, SD 12.8, P=0.000, figure 1). The anxiety levels were comparable to women before IVF treatment, test anxiety and pregnant women after having lost a child to coth death (P≥0.053).

Mothers who had previously experienced a food allergic reaction of their child were more anxious (state anxiety 53.0, SD 14.2) than mothers of children who had not experienced such a reaction (state anxiety 44.7, SD 13.1; P=0.030).

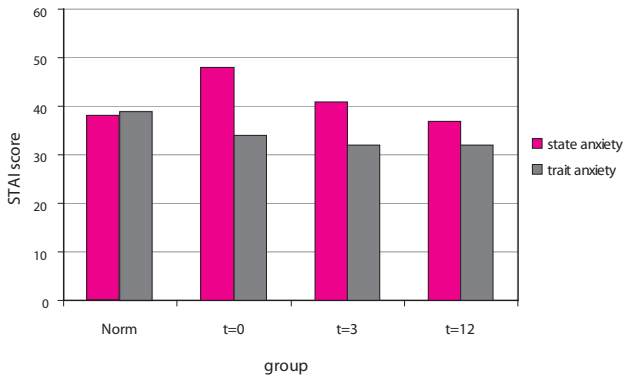
Compared to the fathers, prior to the challenge, mothers were significantly more anxious about a food allergic reaction (mothers 48.9, SD 13.6; fathers 43.9, SD 10.8; P=0.005).

State anxiety after the DBPCFC

Since the trend of anxiety after DBPCFC was comparable between fathers and mothers, only the data relating to mothers is shown in figure 1.

Maternal anxiety about a food allergic reaction of their child (state anxiety) decreased significantly after both a negative and a positive food challenge (P<0.03 for both; table II). The overall decrease in state anxiety of both groups after three months was comparable (P=0.802); the mean decrease in state anxiety in the negative reaction group was 9.9 (SD 16.3) and the mean decrease in the positive reaction group was 10.9 (SD 13.6). One year after the food challenge, the state anxiety was still significantly lower, both in the group with a positive food challenge (mean difference 11.9, P=0.000) and in the group with a negative outcome (mean difference 12.9, P=0.026).

Figure 2.1



The state anxiety (orange bars) and trait anxiety (grey bars) for the norm group and the study group at subsequent time points are displayed. State anxiety levels indicated with (*) are significantly lower than the state anxiety of the patient group at t=0.

Trait anxiety during the study period

During the study period of three months the trait anxiety remained nearly stable (mean difference 1.4, P=0.100, figure 1). The trait anxiety of mothers was significantly lower than the norm group (mean difference mothers and norm group 4.70, P=0.000). No significant difference was found in trait anxiety between mothers who had experienced a previous allergic reaction in their child and mothers without a previous experience (mean difference 2.3, P=0.251).

State anxiety and related factors

Prior to the food challenge, mothers whose child had a positive reaction to the food challenge were already more anxious than mothers whose child had a negative reaction to the food challenge (mean difference=7.1; P=0.053). Mothers of children with a positive DBPCFC had experienced a previous allergic reaction prior to the study more often (56% of positive DBPCFC versus 20% of negative DBPCFC). Linear regression analyses were used to determine related factors to state anxiety (Table III). A previous reaction in their child and trait anxiety were associated with higher levels of state anxiety prior to DBPCFC (B=8.856, P=0.021; B=1.021, P=0.000).

Two weeks after the DBPCFC, the state anxiety in mothers in the positive outcome as well as in the negative outcome group was significantly lower than prior to the food challenge (P<0.01, table II). The outcome of the DBPCFC (positive or negative reaction) was related to state anxiety (B=14.281, P=0.000) at that point in time, whereas a previous reaction prior to the study or trait anxiety were no longer related significantly (B=7.590, P=0.096; B=0.572, P=0.076).

Three months after the DBPCFC only trait anxiety (B=0.801, P=0.000) was associated with state anxiety.

After one year trait anxiety and other food allergies were associated with state anxiety (B=1.108, P=0.003; B=18.074, P=0.028).

Comparison of maternal anxiety between challenge group and refusal group

Comparing the maternal anxiety questionnaires of this refusal group (n=31, mean 50.9, SD 13.5; P=0.346) with those of the challenge group (n=55, mean 47.9, SD 14.2) revealed that there was no significant difference in state anxiety. The refusal group (n=28, mean 39.2, SD 11.1) had a significantly higher trait anxiety than the challenge group (n=54, mean 34.3, SD 7.0; P=0.038). They experienced significant more previous unexpected reactions than the challenge group (74% versus 40%, P=0.002). No differences in demographic variables were found (data not shown).

Table III. Univariable associations of state anxiety at 4 moments

Associated variable	Prior to DBPCFC			2 weeks after DBPCFC		
	B	95% CI (lower bound; upperbound)	P	B	95% (lower bound; upper bound)	P
Type allergy (peanut/hazelnut)	-6.164	(-13.85; 1.53)	0.114	-03.70	(-9.92; 9.18)	0.938
Other food allergies*	3.000	(-11.43; 17.43)	0.678	14.424	(-1.44; 20.29)	0.073
Previous reaction*	**8.856**	**(1.37; 16.34)**	**0.021**	7.590	(-1.41; 16,59)	0.096
Trait Anxiety	**1.021**	**(0.50; 1.54)**	**0.000**	0.572	(-0.06; 1.21)	0.076
DBPCFC outcome (positive/negative)	-	-	-	**14.281**	**(6.79; 21.77)**	**0.000**

Associated variable	3 months after DBPCFC			1 year after DBPCFC		
	B	95% CI (lower bound; upperbound)	P	B	95% (lower bound; upper bound)	P
Type allergy (peanut/hazelnut)	-4.771	(-11.65; 2.11)	0.170	-	-	-
Other food allergies*	12.083	(-0.55; 24.72)	0.060	**18.074**	**(2.12; 34.03)**	**0.028**
Previous reaction*	1.784	(-5.15; 8.72)	0.608	9.562	(-0.24; 19.36)	0.055
Trait Anxiety	**0.801**	**(0.38; 1.23)**	**0.000**	**1.108**	**(0.41; 1.81)**	**0.003**
DBPCFC outcome (positive/negative)	6.178	(-0.48; 12.84)	0.068	8.100	(-2.53; 18.73)	0.130

* yes/no

Discussion

This study showed that before the food challenge parents had high levels of state anxiety about a food allergic reaction in their child. After the food challenges state anxiety was significantly lower while the anxiety disposition remained rather stable troughout the study.

Anxiety is an important psychological measure and an important factor in wellbeing. Many children and parents unnecessarily live with anxiety and elimination diets. A recent meta-analysis showed that about one quarter of American households alter their dietary habits because at least one family member is perceived to have food allergy.[14;15] True food allergy is much less frequent: 5-8% of the children in West Europe.[16] Regardless of the verification by medical expertise, individuals with allergy-like conditions and food hypersensitivity have a lower health-related quality of life.[17] In spite of the lower quality of life among patients with perceived food allergy,[2-6;17] many children are still diagnosed with food allergy based on skin prick tests or IgE levels alone. This practice

overestimates the allergic population.[18;19] In this study, 33% of the children with peanut allergy and 58% of the children with hazelnut allergy had an unnecessary elimination diet.

Food challenges are subject of debate for their suspected burdening impact. The reduced anxiety scores after a food challenge in parents who dare to participate (lower trait anxiety) demonstrate that the opposite might be the case, even among parents of a child with a positive reaction to the DBPCFC.

The parental state anxiety (anxiety about a food allergic reaction of their child) of children with suspected food allergy was high. As far as we know, anxiety about food allergic reactions has never investigated before. Therefore no specific norm groups are available. We compared the parental state anxiety about a food allergic reactions with state anxiety in the refusal group and with other stressful moments, also measured with the STAI. The state anxiety in the challenge group and the refusal group were comparable to women who were pregnant again after losing a baby to coth death[13] and to women just before an IFV treatment.[10] Higher levels of state anxiety were found in parents who had higher levels of trait anxiety and in parents who experienced one or more unexpected food allergic reactions in their child prior to the study.

The trait anxiety in the challenge group is lower than the norm group while the trait anxiety of the refusal group is comparable with the norm group, this suggests a sample bias because of the voluntary participation. It also suggests that parents with a lower trait anxiety participate while parents with a normal or higher trait anxiety refuse a DBPCFC.

Both after a negative and a positive DBPCFC, the state anxiety of parents was significant lower. The decreased state anxiety was still present after one year. The trait anxiety of parents remained unchanged throughout the study period, which shows that trait anxiety is a stable, individual disposition.

Two weeks after the food provocation, the outcome (positive or negative reaction) of the DBPCFC was strongly related to state anxiety. At this time point, no relation between state and trait anxiety was shown. It may be that shortly after the DBPCFC the impact of the outcome was so strong that trait anxiety had no influence. Nevertheless, this result should be carefully interpreted, because a smaller, potentially biased group of parents sent back the anxiety questionnaire after 2 weeks. Three months and one year after the food challenge, with a small number of individuals lost to follow up, trait anxiety was significantly related again to the state anxiety as well. After one year other food allergies turned out to be significantly related to higher state anxiety. This is in line with the study of Cohen et all, in which was found that parents whose children had multiple food allergies had a lower quality of life than whose children had fewer allergies.[4]

The lower state anxiety after DBPCFC can be discussed. It is argued that repeated administration of a measure of psychological function might result in regression towards the mean, even without interventions. Pre-post measures of state anxiety described by Van der Ploeg et all varied from no differences (pre and post IVF treatment) to a decrease of 2 (pre and post operation) to 6 points (control group test anxiety). This suggests that the mean decrease of 10.5 points in our study group is not only explained by regression towards the mean.

Our data may suggest that the DBPCFC is not only a diagnostic tool in food allergy; it might also have a therapeutic influence on anxiety reduction in food allergy. A plausible explanation for the decrease of anxiety in both positive and negative food challenge groups could be the psychological effect of reducing anxiety by exposure to the feared stimulus.[20] It is known that anxiety is reduced by exposure in a controlled situation, and by learning appropriate skills for dealing with the stressor and the person's own anxiety.[21] In our study, a food challenge in the presence of a doctor and in a clinical setting gave the opportunity for exposure to the feared stimulus in a controlled situation. Parents learned to identify the onset of the symptoms and how to give appropriate treatment for the food allergic reaction modeled by the doctor. Modeling is a therapeutic technique in which the therapist – in this case the doctor – demonstrates how to approach the stressor.[20]

In dialogue with parents, we noticed that they often believed that food allergic reactions occur with no warning symptoms after ingestion of peanut or hazelnut, and that their child would immediately pass away. Although parents were told that mild reactions may progress into severe ones, they were able to experience a sense of control in case of a future food allergic reaction. Perceived feelings of control play an important role in anxiety. This was also found in children with peanut allergy: they felt safer when carrying epinephrine kits.[5]

Conclusion

Parents show high levels of state anxiety about a food allergic reaction in their child. Lower state anxiety levels in the parents after DBPCFC suggests that besides this procedure is the 'gold standard' in food allergy diagnosis, it also might be succesful in reducing parental anxiety about a food allergic reaction.

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Clinical reactivity to hazelnut in children:
association with sensitization to birch pollen or nuts?

Abstract

Background: Sensitization to hazelnut can be detected early in life, even without previous known oral exposure to hazelnut.

Objective: To determine clinical reactivity to hazelnut and eliciting doses (ED) in sensitized children by double-blind placebo-controlled challenge (DBPCFC) and relate this to sensitization to birch pollen, other nuts and peanut.

Methods: Twenty-eight sensitized children ≥ 4 years with suspected hazelnut allergy were evaluated by DBPCFC comprising 9 doses ranging from 10 µg to 3 gram hazelnut and a final dose of 10 hazelnuts. Skin prick tests (SPT) with commercial hazelnut extract was performed and specific IgE to pollen, hazelnut, other nuts, and peanut was determined.

Results: Doses up to 1 mg were tolerated by all. Sixteen children (57%) had no allergic reaction to hazelnut during challenge. Four children reported only OAS on doses starting from 10 mg. Eight children developed additional objective symptoms (≥ 300 mg). SPT reactivity to hazelnut and hazelnut-specific IgE levels were significantly higher in children with objective reactions. Children with objective reactions were consistently sensitized to all nuts and peanut and specific IgE levels were significantly higher than in children without objective reactions. Three children with objective reactions to hazelnut had low or undetectable birch pollen-specific IgE.

Conclusion: Sensitization to hazelnut was shown to be clinically relevant in 43% of the children tested. All tolerated 1 mg. Most reactions to hazelnut consisted of OAS and additional objective symptoms, which were not always accompanied by sensitization to birch pollen, but always by sensitization to other nuts and peanut.

Introduction

Children can be sensitized to hazelnut at an early age. [1] Remarkably, a large proportion has never to their knowledge ingested hazelnut and most allergic reactions to nuts in children occur after the first known exposure. [2] The route to hazelnut sensitization in childhood has yet to be elucidated. In areas where birch trees are endemic, such as northern Europe and America, hazelnut allergy in adults is often a result of primary sensitization to birch pollen allergen Bet v 1, which is cross-reactive with the homologous allergen in hazelnut Cor a 1. [3] Birch pollen related hazelnut allergy is usually mild and remains restricted to the oral cavity. [4] Oral allergy to hazelnut has been described as symptom in pollen-sensitized children. [5; 6] This contrasts with the reported serious and sometimes life-threatening reactions in children after hazelnut ingestion. [7]

Sensitization to hazelnut in early childhood has been related to sensitization to other tree nuts and peanut. [1; 8] These allergens can also cause serious reactions, [9] which may suggest a mutual route of sensitization to these allergens.

To investigate the relevance of hazelnut sensitization in childhood and its relation to other sensitizations, it is important to establish a clear diagnosis of hazelnut allergy by double-blind placebo-controlled food challenge (DBPCFC). Previous studies in children only presented data on allergic reactions to hazelnut according to case history and not from DBPCFC. Moreover, there are no data on eliciting doses of hazelnut in children. Because hazelnut can be a hidden food allergen in a variety of food products, such data are useful in determining the level of contamination with hazelnut in food products in order to prevent serious allergic reactions. [10; 11]

The aim of our study was to determine the clinical reactivity to and eliciting doses (ED) of hazelnut by DBPCFC in sensitized children. We also studied the sensitization to birch pollen and other nut species and peanut in this group.

Methods

Patients

Twenty-eight children (8 female, 20 male; age 4 – 16 years) with suspected allergy to hazelnut were recruited from the outpatient clinic of the Department of Pediatric Dermatology and Allergology between June 2004 and September 2005. All children were sensitized to hazelnut (specific IgE $\geq$ 0.35 kU_A/l and/or SPT $\geq$ 0.5) and had either experienced a previous reaction to hazelnut or had never to their knowledge ingested hazelnut. Elimination diets for hazelnut and other tree nuts were

initiated prior to the challenge. Parents completed an extensive questionnaire on atopic symptoms, elimination diet, and previous allergic reactions to hazelnut, other nuts and peanut. The severity of atopic dermatitis for each child was calculated using the mean SCORAD of three time points (before, during and 3 months after the challenge). [12] All parents gave written informed consent before enrolment in the study. The study was reviewed and approved by the Central Committee on Research investigating Human Subjects in The Netherlands.

Skin prick test

Skin prick tests were performed on the patient's back with a prick needle and standardized hazelnut extract (ALK-Abelló, Nieuwegein, The Netherlands). Prior to the skin tests, patients discontinued antihistamines. 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. The wheal reaction was measured after 15 minutes and transferred with transparent adhesive tape onto a record sheet. The area of the skin wheal was determined by computer scanning. [13] SPT responses were standardized by dividing the wheal area of the hazelnut prick by that obtained for the histamine control. Ratios ≥ 0.5 were considered positive.

Specific IgE levels

Specific IgE levels prior to DBPCFC were determined using the CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Besides hazelnut, coexisting sensitization to birch pollen, almond, pecan, walnut, Brazil nut, pistachio, cashew and peanut was also investigated by the CAP system FEIA. IgE levels ≥ 0.35 kU_A/l were considered positive. All serum samples were checked for IgE reactivity to Cross-reactive Carbohydrate Determinants (CCD) using the Radio Allergo-Sorbent Test (RAST). [14]

DBPCFC hazelnut

Challenges were performed as described previously. [15] Briefly, children were only challenged when they were otherwise healthy and had no exacerbation of atopic diseases. DBPCFC were not performed in the tree pollen season between mid-February and mid-June. Medication (topical steroids, inhalers, antihistamines) was stopped before the challenge. None used β–blocking agents, angiotensin converting enzyme inhibitors or immunosuppressive agents. After intravenous access was assured, 9 portions of defatted hazelnut flour were administered in series: 10 µg, 100 µg, 500 µg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g, and 3 g (protein content 15.5%; kindly provided by the

Table I. Characteristics of the study group (n=28).

Characteristic	Number (%)
Sex	
Female	8 (29%)
Male	20 (71%)
Mean age	7.5 years
Atopic history	
Atopic dermatitis	24 (86%)
Seasonal rhinoconjunctivitis	16 (57%)
Allergic asthma	13 (46%)
Previous reactions to hazelnut	
At least one	4 (14%)
None recalled	24 (86%)

Food Allergy Research and Resource Program, University of Nebraska, USA). Portions were masked in whole-wheat instant cereal and applesauce (a pinch of cinnamon powder was added to improve masking). Four placebos were randomly interspersed by the pharmacist, in order to complete the procedure in one day. The last dose was unblinded and consisted of 10 hazelnuts (5 gram; ca 635 mg protein), because this amount could not be masked in edible portions for children. The doses were given in time intervals of 15 - 30 minutes. After reporting subjective symptoms (OAS, abdominal pain), the next dose was postponed until the symptoms resolved. The challenge was discontinued when an objective reaction occurred (urticaria, angio-edema, rhinoconjunctivitis, vomiting, diarrhea, bronchoconstriction, or hypotension). Objective symptoms were treated with appropriate medication. [16] To document possible late reactions, all parents were contacted by phone the day after the challenge.

Statistics

SPT and specific IgE were not normally divided, even after logarithmic transformation. Therefore, all calculations were done with non-parametric tests. The severity of hazelnut allergy was stratified to three groups according to the reaction during challenge: no reaction, OAS, and objective reaction. The Mann Whitney U test was used to calculate differences in SPT and specific IgE between the different types of reaction. Correlation between different specific IgE levels was calculated with Spearman's rank correlation coefficient. Differences were considered significant if the p-value was < 0.05. Statistical analysis was performed using the Statistical Program SPSS (version 12.0, SPSS Inc., 2001, Chicago USA).

Results

Study population

The clinical characteristics of the 28 participating hazelnut-sensitized children are displayed in Table I. All children were atopic, with atopic dermatitis as the most frequently reported condition (86%). Sixteen children (57%) suffered from seasonal rhinoconjunctivitis. Asthma related symptoms were reported by 13 children (47%). Atopic dermatitis was not associated with a higher prevalence of either seasonal rhinoconjunctivitis or asthma, but seasonal rhinoconjunctivitis was correlated with asthma (p=0.049).

Determination of clinical reactivity and eliciting doses of hazelnut

DBPCFC were performed on 28 children. Doses up to 1 mg (0.16 mg protein) were tolerated by all (Table II).

Sixteen children (57%) did not show any reaction upon hazelnut ingestion during the challenge procedure. One child in this group (Ha 21) developed several hives on the face after skin contact, but oral ingestion did not elicit any further symptoms.

In 12 children (43%) hazelnut allergy was confirmed by challenge. Four children (4/12; 33%) reported OAS without developing objective symptoms. The interval between OAS and ingestion was always less than 10 minutes. One child (Ha 22) reported OAS at 10 mg and after all subsequent hazelnut doses up to 10 hazelnuts. Another child (Ha 11) reported aggravating symptoms of OAS at doses ranging from 100 mg – 1 gram and refused further portions, resulting in discontinuation of the challenge. Two children (Ha 12 and Ha 23) had a typical cough associated with tingling of the mouth and throat after the last dose of 10 hazelnuts, categorized as OAS.

Eight children (8/12; 67%) developed an objective reaction to hazelnut. ED for objective reactions ranged from 300 mg to 10 hazelnuts. In five children the objective reactions were preceded by OAS on previous doses (100 mg – 3 gram), in three children OAS and objective symptoms developed on the same dose. Abdominal pain was reported by 6 children 15-30 minutes after ingestion, resulting in vomiting (n=4) or diarrhea (n=1) 50-90 minutes after ingestion. Systemic urticaria (n=3) started with an uncomfortable child, and the typical hives spread over the skin between 40 and 120 minutes after the last dose. One child developed bronchoconstriction after 40 minutes. All 8 children with objective symptoms received antihistamines and steroids and could be discharged in good condition the same day. Late reactions were not reported.

According to the results of the challenge, children were divided into three groups: children without

reaction (n=16), children with only OAS (n=4) and children with additional objective reactions (n=8).

Hazelnut allergy in relation to atopy

Children without reaction to hazelnut had similar levels of total IgE as children with objective reactions. Children with only OAS had significantly lower total IgE levels than children with objective reactions (p=0.028). The mean SCORAD (before, during and 3 months after the challenge) was similar in children without reaction, with only OAS and with objective symptoms (data not shown).

Hazelnut allergy in relation to previous reactions

Four children (14.3%; Ha 21, Ha 11, Ha 8 and Ha 28) experienced a previous allergic reaction that was clearly related to hazelnut according to their history. These four children described symptoms after ingestion of granola, chocolate or custard containing hazelnut. OAS was reported as the only symptom in one child (Ha 11) while three had multiple objective symptoms (urticaria, facial swelling, rhinoconjunctivitis, vomiting, diarrhea, and/or dyspnea). During the challenge, three of the four children who had experienced a previous allergic reaction to hazelnut developed similar symptoms, whereas one child did not respond (Ha 21). This child appeared to have outgrown hazelnut allergy.

Hazelnut allergy in relation to hazelnut sensitization

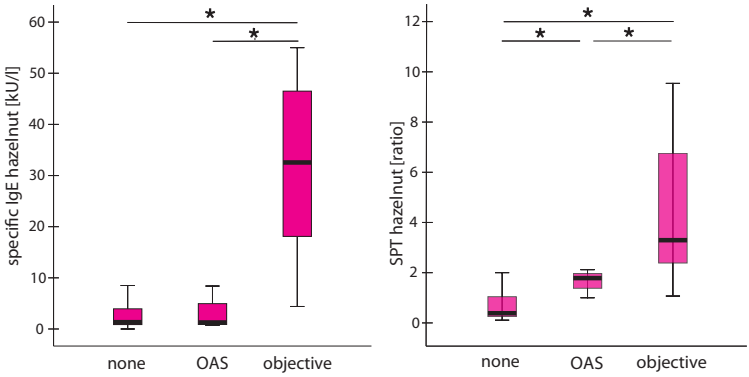
Sensitization was re-evaluated prior to the challenge, (Table II). Two children (Ha 18 and Ha 20) with a positive SPT but without hazelnut-specific IgE at this time had elevated IgE in the past (0.98 and 1.63 kU_A/l, respectively).

The group with objective reactions had significantly higher SPT reactivity (median ratio 3.3; range 1.1 – 22.6) and specific IgE levels (median 32.5 kU_A/l; range 4.4 - 55) compared to the other two groups (Table II, Figure 1). In addition, SPT reactivity (median ratio 1.8; range 1 – 2.1), but not specific IgE (median ratio 0.4; range 0.1 – 2.0) was significantly higher in the OAS group than in the group without reaction.

Hazelnut allergy in relation to pollen allergy and sensitization

Table III shows that the proportion of children with sensitization to birch pollen, as well as the proportion of children with seasonal rhinoconjunctivitis, was similar between children without hazelnut allergy (median 46 kU_A/l, range <0.35 – 100), with OAS (median 39 kU_A/l, range 2.6 – 80),

Figure 3.1



Median SPT reactivity (A) and specific IgE levels to hazelnut (B) with interquartile ranges in relation to the reaction during challenge (none, OAS, objective). Differences between the groups are calculated with Mann Whitney U, and significance is indicated as * (P<0.05).

and with objective reactions to hazelnut (median 22 kU_A/l, range <0.35 – 100). Levels of grass pollen-specific IgE did not differ between the groups either.

Four children without a reaction to hazelnut had undetectable birch pollen-specific IgE and also undetectable or low hazelnut-specific IgE (<0.35 – 1.3 kU_A/l). All children with only OAS were sensitized to birch pollen. Three of them had symptoms of rhinoconjunctivitis, two of whom could clearly relate this to the birch pollen season. Three of the eight children with objective reactions to hazelnut had low or undetectable birch pollen-specific IgE (<0.35 - 0.5 kU_A/l) compared to the levels of hazelnut-specific IgE (27-55 kU_A/l). Grass-pollen IgE was also low or undetectable (<0.35 – 1.0 kU_A/l) in these children.

Hazelnut allergy in relation to sensitization to other nuts and peanut

Because sensitization to hazelnut in childhood can be associated with sensitization to other nuts and peanut, we investigated specific IgE to various common tree nuts and peanut (Table III). Sensitization to at least two other nuts (almond, pecan, walnut, Brazil nut, pistachio and/or cashew) was present in 22 children (79%). Sensitization to peanut was present in 27 children (96%).

The group of children with objective reactions was sensitized to more species of nuts (median 6; range 5 – 6) than the group of children with only OAS (median 4.5; range 0 – 6) or no reaction (median 2.5; range 0 – 6). Furthermore, specific IgE to all tested nuts was significantly higher in the group with objective reactions compared to the group without reaction (p< 0.05 for all nuts). The difference between specific IgE to peanut in children with and without objective reaction to

Table II. Hazelnut sensitization and symptoms during DBPCFC (n=28).

		Hazelnut		DBPCFC Hazelnut		
No	Age	IgE*	SPT†	Symptoms‡	ED OAS§	ED objective§
ha 6	8	8.5	0	none	-	-
ha 9	9	2.0	0.9	none	-	-
ha 10	14	1.4	1.5	none	-	-
ha 13	8	3.7	0.	none	-	-
ha 15	16	1.0	0.5	none	-	-
ha 16	14	26	0	none	-	-
ha 17	7	1.1	0.9	none	-	-
ha 18	4	0	2.0	none	-	-
ha 19	4	1.3	0	none	-	-
ha 20	6	0	0.5	none	-	-
ha 21	7	3.5	1.2	none	-	-
ha 24	5	11	0	none	-	-
ha 26	4	0.7	0	none	-	-
ha 29	7	1.3	0	none	-	-
ha 33	6	4.2	1.2	none	-	-
ha 34	4	0.5	0	none	-	-
ha 11	12	8.4	2.1	oas	100	-
ha 12	4	1.5	1.8	oas	>3000	-
ha 22	10	1.0	1.0	oas	10	-
ha 23	5	0.7	1.8	oas	>3000	-
ha 7	4	16	2.8	oas, ap, vom	>3000	>3000
ha 8	6	4.4	1.1	oas, urt	100	>3000
ha 25	8	45	2.0	oas, rc, ap, vom	3000	>3000
ha 27	13	20	3.8	oas, urt, ap	100	>3000
ha 28	4	38	9.5	oas, ap, vom	100	300
ha 30	8	48	23	oas, ap, vom	100	3000
ha 31	6	27	2.7	oas, urt, rc, bro	>3000	>3000
ha 35	6	55	4.0	oas, ap, dia	>3000	>3000

* IgE in kU_A/l; 0: <0.35; 100: >100

† skin prick test (SPT) in area hazelnut / area histamine; 0: < 0.5

‡ symptoms:
oas=oral allergy syndrome
urt=urticaria
rc=rhinoconjunctivitis
ap=abdominal pain
vom=vomiting, dia=diarrhea
bro=bronchoconstriction

§ Eliciting doses (ED) in mg

hazelnut was borderline significant (p=0.061). Hazelnut-specific IgE was highly correlated with IgE to pecan (r=0.83; p<0.001), almond (r=0.77; p<0.001), Brazil nut (r = 0.75; p<0.001), walnut (r=0.74; p<0.001), and to a lesser extent to pistachio (r=0.62; p=0.001), cashew (r=0.57; p=0.002) and peanut (r=0.52; p=0.004). To rule out that this was due to cross-reactivity to carbohydrate determinants of glycoproteins (CCD), IgE binding to CCD was determined in all children. This was negative in all but one patient (Ha 23; data not shown) and was not correlated with the type of reaction.

Eleven children had a history of one (n=9) or more (n=2) allergic reactions (more than OAS) to other species of nuts than hazelnut or peanut. Most reactions were related to the ingestion of cashew (n=5) and peanut (n=6).

Discussion

Twelve hazelnut-sensitized children (43%) developed a reaction during challenge, either only OAS (33 %) or OAS with additional objective symptoms (67 %). OAS was reported after doses of 10 mg or higher, whereas objective symptoms developed upon 300 mg or higher. Objective reactions were associated with significantly higher SPT reactivity to hazelnut and specific IgE levels to hazelnut and other nuts compared to children without objective reactions, but no difference in sensitization to birch pollen was observed.

Challenges are an important tool in diagnosing food allergy. [17] In this study, fewer than half of the children with sensitization to hazelnut appeared to be hazelnut allergic. Moreover, one child in this study with a previous reaction to hazelnut did not react further, suggesting resolution of hazelnut allergy. [18]

Challenges with increasing doses of hazelnut are the only way to determine ED. To our knowledge, this is the first study to report ED for hazelnut in children. Doses up to 1 mg hazelnut flour were tolerated by all, which is the no observed adverse effect level (NOAEL) in this study. The ED for OAS was ≥ 10 mg (1.6 mg protein); the first objective symptoms developed after 300 mg (46.5 mg protein). This is in line with a previous study on hazelnut allergy in adults. [19] In a recently published comparable study of peanut-sensitized children we found an ED for objective symptoms of 100 mg peanut flour (50 mg protein). [15] Other studies in adults described ED up to 15 gram hazelnut (ca. 1.5 gram protein), without mentioning the type of symptoms. [20; 21] The relatively high doses necessary to provoke symptoms found in previous studies are in accordance with our finding that five children developed the first symptoms after the last dose of 10 hazelnuts (ca. 635

mg protein). Apparently the ED for hazelnut is comparable between adults and children.

Sixty-seven % of children with hazelnut allergy had objective symptoms, such as systemic urticaria, rhinoconjunctivitis, vomiting and dyspnea. This contrasts with the small percentage of objective reactions found in adults (7%) from our hospital. [19] It is unlikely that this is the result of a selection bias since the referral area is similar. This probably suggests that adults and children experience a real difference in the type of reaction to hazelnut. Adults from mid and northern Europe usually experience OAS to hazelnut as major symptom without objective symptoms, which has been related to cross-sensitization to the major birch-pollen allergen Bet v 1. [3] Only few studies described OAS to cross-reactive foods in birch-pollen sensitized children, but these data were not confirmed by oral challenges. [5; 6] In our study, children with only OAS were all sensitized to birch pollen, although only half reported symptoms of rhinoconjunctivitis during the birch pollen season. In contrast, three of the children with additional objective symptoms to hazelnut had low or even undetectable birch pollen or grass pollen specific IgE. This suggests a non-pollen related route of sensitization to hazelnut in some of the children from areas where birch pollen is endemic.

Sensitization to hazelnut in childhood can be accompanied by sensitization to many other nuts. [1; 22; 23] In adults, cross-sensitization between hazelnut and other tree nuts and peanut has been suggested as well, although less often than in children. [24] In our study, high levels of specific IgE to other nuts were seen particularly in children with objective reactions to hazelnut. These children also had high levels of peanut-specific IgE, but the difference with non-allergic children was less pronounced. The differences in sensitization to nuts and peanut between children with and without objective reactions to hazelnut did not appear to be explained by the extent of atopy, because no differences in total IgE levels and SCORAD were observed. The effect of cross-sensitization based on reactivity to CCD has been ruled out in this study population. [14] Although we did not investigate the clinical relevance of all nuts and the history did not provide information on previous exposure to most nuts, avoidance of all nuts may still be advisable in children with objective reactions to hazelnut.

For future preventive and therapeutic strategies, it would be very interesting to investigate which major allergens are responsible for the primary sensitization in children with objective reactions to hazelnut. For example, non-pollen-related allergens such as Cor a 8 (lipid transfer protein, LTP) or Cor a 9 (legumin, an 11S globulin), which have been implicated in the induction of systemic reactions to hazelnut, may be interesting. [25-27]

Table III. Sensitization to pollen, tree nuts and peanut.

Pt nr	Total IgE	Grass	Birch	Hazelnut	Almond	Pecan
ha 6	1734	100	100	9	1	1
ha 9	435	49	76*	2	0	0
ha 10	844	100	56*	1	1	0
ha 13	2225	100	100*	4	0	-
ha 15	626	1	0	1	0	0
ha 16	1881	18	39*	26	19	1
ha 17	388	0	27	1	0	0
ha 18	32	0	0	0	0	-
ha 19	-	21	53	1	0	1
ha 20	268	0	0	0	0	0
ha 21	1485	7	100*	4†	1	1
ha 24	4433	58	100*	11	7	6
ha 26	292	68	0	1	0	0
ha 29	2026	24	1.6*	1	0	0
ha 33	2313	100	100	4	0	1
ha 34	553	0	0.4	0	0	0
ha 11	512	7	51	8†	1	36
ha 12	362	26	27*	2	3	3
ha 22	580	55	80*	1	0	0
ha 23	30	2	2.6	1	0	0
ha 7	1116	32	9.1*	16	3†	2
ha 8	1536	3	35	4†	1	22
ha 25	2459	60	100*	45	11	32
ha 27	1550	34	62*	20	9	7
ha 28	288	1	0	38†	2	22
ha 30	1985	7	100	48	7	13
ha 31	2208	0	0.5	27	0	17
ha 35	4171	0	0	55	12	3

All values are in kU_A/l

* Rhinoconjunctivitis during the birch pollen season
† Allergic reaction after accidental ingestion

Conclusion

As can be expected in an area where birch pollen is endemic, OAS was reported by all children with a reaction to hazelnut. ED for OAS were ≥ 10 mg hazelnut flour. Additional objective symptoms were observed in at least 67% of the reactions, with ED ≥ 300 mg. These objective reactions to hazelnut were not always accompanied by sensitization to birch pollen, but sensitization to other nuts and peanut was observed in all children with objective reactions to hazelnut.

Pt nr	Hazelnut	Walnut	Brazil	Pistachio	Cashew	Peanut
ha 6	9	1	1	4	2	10†
ha 9	2	0	0	0	0	4†
ha 10	1	0	0	1	0	1
ha 13	4	0	-	1	0	15
ha 15	1	0	1	0	0	87†
ha 16	26	0	2	20	20	100†
ha 17	1	0	0	3	3	9
ha 18	0	0	-	-	4†	0
ha 19	1	1	0	6	3	12
ha 20	0	0	0	4	4†	1†
ha 21	4†	1	1	6	3†	8
ha 24	11	4	8	100	100	48
ha 26	1	0	1	1	2	2
ha 29	1	0	1	3	3	3
ha 33	4	0	1	1	0	1
ha 34	0	0	0	0	0	3
ha 11	8†	26	3	6	5	4
ha 12	2	3	2	5	2	7
ha 22	1	0	1	41	27	25†
ha 23	1	0	0	0	0	0
ha 7	16	1	7	10	8	40
ha 8	4†	14	0	21	28	66
ha 25	45	23	10	56†	59†	52
ha 27	20	4	-	18	15	100
ha 28	38†	22	3	6	6	3
ha 30	48	12	18	27	27	50
ha 31	27	4	10	1	6	2
ha 35	55	1	37	100	100†	66

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Lipid transfer protein (LTP)-linked food allergies are not limited to the Mediterranean area:
Cor a 8 sensitization in hazelnut-allergic children from a birch endemic area.

Abstract

Background: Hazelnut allergy in birch pollen-exposed areas is usually cross-reactive (Cor a 1 and 2) and mild in nature (oral allergy). In areas without birch trees, severe reactions are more prevalent and linked to sensitization to lipid transfer protein (LTP), Cor a 8.

Objective: To investigate whether sensitization to LTP plays a role in more severe (objective) hazelnut-induced symptoms in children from a birch-endemic area.

Methods: Sensitization to Cor a 8, Cor a 2, Cor a 1 and Bet v 1 was determined by RAST and immunoblot in hazelnut-sensitized children, with (n=8) and without (n=18) objective reactions during double-blind placebo-controlled food challenge (DBPCFC). Additionally, samples from 191 hazelnut-sensitized non-challenged children were analyzed.

Results: Children with objective reactions during DBPCFC had higher IgE titers to hazelnut ($p<0.001$) and recognized more allergens on immunoblot ($p=0.001$) than those without. All children with objective symptoms were sensitized to Cor a 8 (0.51-23.3 IU/ml), compared to only one child (1/18) without objective reactions (0.90 IU/ml). In a univariate analysis, IgE against Cor a 2 and Cor a 8 was associated with objective symptoms, but in multivariate analysis only IgE against Cor a 8 remained as independent risk factor (undefined OR; $p<0.0001$). In the group of non-challenged children (n=191), prevalence of LTP sensitization was >30%. Unexpectedly, sensitization to Cor a 1 was observed in children that were not sensitized to Bet v 1.

Conclusion: Sensitization to hazelnut LTP is a strong risk factor for objective symptoms during DBPCFC in children from a non-Mediterranean birch-endemic area.

Introduction

In areas where the birch is endemic, hazelnut allergy has mainly been associated with cross-reactive IgE to Bet v 1 and Bet v 2 (profilin), usually causing mild symptoms known as the oral allergy syndrome (OAS). [1-3] The cross-reactive structures in hazelnut implicated in this so-called para-birch syndrome are Cor a 1 and Cor a 2. In Mediterranean areas without exposure to birch pollen, reactions to hazelnut have been associated with sensitization to Cor a 8, a lipid transfer protein (LTP).[4] Sensitization to LTP has convincingly been shown to be a risk factor for severe allergic reactions in these areas, not only to hazelnut [4] but in particular also to apple and peach. [5;6] In northern European countries like The Netherlands, [5] Austria [5] and Germany [7] sensitization to LTP has only rarely been reported for patients with plant food allergies and most of these studies were performed among adults. Although patients from birch pollen-endemic areas predominantly present mild oral allergy to hazelnut, severe symptoms can occur, and even fatal reactions to hazelnut have been reported. [8-10] Many of the more severe reactions were found in children. Recently, in a study among Dutch children with DBPCFC-proven hazelnut allergy, we reported that 8 out of 12 developed challenge-induced objective symptoms (generalized urticaria, angio-oedema, vomiting and bronchoconstriction) on top of mild oral symptoms reported by the whole group. [11] Remarkably, three of the eight children with objective reactions had no or very low IgE levels to birch (or grass) pollen, suggesting that the origin of sensitization was not pollen cross-reactivity. Such a pattern of fruit and/or nut allergy in the absence of, or at least not necessarily linked to pollen allergy, is typical for LTP-related allergy in Mediterranean countries like Spain [12] and Greece. [13] Children frequently report first symptoms of hazelnut allergy after their first known oral exposure. [9;14] The route of sensitization is still largely a mystery, but is likely to be different from pollen-induced cross-reactivity.

The aim of this study was to investigate whether sensitization to hazelnut LTP (Cor a 8) plays a role in children from birch endemic areas presenting with more severe hazelnut allergy. This may challenge the dogma that has taken foothold in the scientific community that sensitization to LTP is a Mediterranean problem.

Methods

Study population

Sera from 26 children with specific IgE to hazelnut (8 female and 18 male; median age 7.0 years)

were collected between January 2003 and June 2004. These children belonged to a group of children that underwent a DBPCFC with increasing doses of hazelnut. Sera used in the present study were taken prior to the challenge. The outcome of the DBPCFCs was reported elsewhere. [11] Of the 26 challenged children, 14 were shown to be negative in the challenge, four had only OAS and eight additionally demonstrated objective symptoms (angio-oedema, generalized urticaria, vomiting, diarrhea, rhinoconjunctivitis and/or dyspnea). [11] To identify risk factors for objective symptoms, the subjects without symptoms (n=14) and with only OAS (n=4) were analyzed together (n=18) and compared to those with objective symptoms (n=8).

In the same period, all children (between 0.4 and 16.6 years of age) visiting the outpatient clinic for pediatric allergology (n=1290) were routinely screened for sensitization to various food allergens, including hazelnut, by the CAP-FEIA system (Phadia, Uppsala, Sweden). Of them, 310 children (24%) were sensitized. Remaining serum after determination of hazelnut-specific IgE was stored at -20ºC. In total, 191 serum samples from children with sensitization to hazelnut were available for further analyses in this study. This group was not different from the total group (n=310) with respect to sex (67 female and 124 male vs. 115 female and 195 male), age (median age 4.5 vs. 4.1 years) and hazelnut-specific IgE (median 2.4 vs. 2.2 kU/L).

Determination of hazelnut specific IgE by CAP

Levels of specific IgE to hazelnut were determined using the CAP-FEIA system according to manufacturer's instructions. IgE levels of more than 0.35 kU/L were considered positive.

Determination of specific IgE by Radio-allergosorbent test (RAST)

RAST was performed as described previously. [15] In short, purified natural (n) Bet v 1, nCor a 1, recombinant (r) Cor a 1 (rCor a 1.0401, kindly provided by Stefan Vieths, Langen, Germany) [16], nCor a 8 or rPru p 3 [17] was coupled to 100 mg CNBr-activated Sepharose (GE Healthcare, Uppsala, Sweden). nCor a 1 and nBet v 1 were both purified by means of affinity purification using monoclonal antibody 5H8 as described elsewhere. [18] nCor a 8 was purified by means of a combination of ion exchange and size-exclusion chromatography similar to that described for apple LTP. [19] Purity of nCor a 1 and nCor a 8 was assessed by SDS-PAGE and silver staining (fig.1). RAST analysis for profilin (Cor a 2) was performed using Sepharose-coupled poly-L-proline as affinity matrix to bind Cor a 2 from hazelnut extract. After washing away unbound hazelnut protein, this solid phase was used for detecting Cor a 2-specific IgE. [20] RAST was carried out by overnight incubation of 50 µl serum with 0.5 mg Sepharose in a final volume of 300 µl PBS / 0.3% BSA / 0.1% Tween 20

(PBS-AT). After washing away unbound material with PBS / 0.1% Tween 20 (PBS-T), bound IgE was detected by overnight incubation with ^{125}I-radiolabelled sheep-anti human IgE (SH25-1-p7; Sanquin, Amsterdam, The Netherlands). After washing, bound radioactivity was measured in a -counter. Results were expressed in international units (IU) IgE per ml using a standard curve of chimeric monoclonal IgE antibodies against Der p 2 and Sepharose-coupled rDer p 2. [21] Result > 0.35 IU/ml were considered positive.

SDS-PAGE and immunoblotting with hazelnut extract

Crude hazelnut extract (20 µg/cm total protein) or purified nCor a 8 (15 µg/cm) was separated by SDS-PAGE under reducing conditions using NuPAGE-Bis-Tris gels (10%, Novagen, Groningen, the Netherlands) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), following the manufacturer's instructions. For immunoblot analysis, serum samples were diluted 1:30 in PBS containing 0.05% Tween 20. Serum from a non-atopic subject was used as negative control. For detection of bound IgE antibodies, the strips were incubated with ^{125}I-radiolabeled sheep anti-human IgE (Sanquin).

Statistics

For statistical analyses cut-off values for sensitization to individual allergens were established by ROC-analysis instead of using the standard assay cut-off of 0.35 IU/ml. For the ROC-curve analysis, objective symptoms during DBPCFC were classified as positive and OAS and negative symptoms as negative. The resulting cut-off values for each individual allergen were used in a univariate analysis. The Fisher's exact test (two-way analysis) was used to establish associations between sensitization to individual allergens and objective symptoms during challenge. For age and challenge outcome, the Mann-Whitney U test was used. Significant associations ($p < 0.15$) from the univariate analyses were analyzed in a multivariate analysis using a logistic regression model. Correlations between sensitization to different allergens were performed with the non-parametric Spearman's rank or Gamma test. All calculations were performed using SPSS (version 12, SPSS Inc., 2001, Chicago, USA). P-values <0.05 were considered significant.

Results

Sensitization to hazelnut LTP (Cor a 8) is a risk factor for objective symptoms

Hazelnut allergens Cor a 8 and Cor a 1 were purified from hazelnut extracts and shown to be 99%

Table I. Sensitization to hazelnut, birch pollen, nBet v 1, nCor a 1, rCor a 1, nCor a 2, nCor a 8 and rPru p3 in children who were challenged with hazelnut. Differences in levels of specific IgE and number of recognized allergens between children with and without objective symptoms are indicated with a p-value on the bottom of each column.

Children		DBPCFC				
pt	sex*	allergy†	age‡	hazelnut§	birchpollen§	Bet v 1§
ha 6	M	none	8	8.5	>100	86.8
ha 9	F	none	9	2.0	76.0	12.7
ha 10	M	none	14	1.4	56.0	18.4
ha 13	M	none	8	3.7	>100	39.7
ha 15	M	none	16	1.0	neg	neg
ha 16	M	none	14	26.1	39.0	13.8
ha 17	M	none	7	1.1	26.7	5.7
ha 19	M	none	4	1.3	53.0	15.4
ha 21	M	none	7	3.5	>100	49.4
ha 24	M	none	5	11.0	>100	87.5
ha 26	M	none	4	0.7	neg	neg
ha 29	F	none	7	1.3	1.6	neg
ha 33	M	none	6	4.2	>100	37.3
ha 34	F	none	4	0.5	neg	6.2
ha 11	M	OAS	12	8.4	51.0	4.2
ha 12	F	OAS	4	1.5	27.4	neg
ha 22	F	OAS	10	1.0	80.0	16.6
ha 23	M	OAS	5	0.7	2.6	0.7
ha 7	M	object	4	16.1	9.1	13.5
ha 8	M	object	6	4.4	34.9	5.9
ha 25	M	object	8	45.0	>100	56.6
ha 27	M	object	13	20.1	62.0	19.4
ha 28	F	object	4	38.0	neg	neg
ha 30	F	object	8	48.0	>100	76.3
ha 31	F	object	6	27.1	0.5	neg
ha 35	M	object	6	55.0	neg	neg
				P<0.001	P=0.567	P=0.567

*sex: M=male, F=female

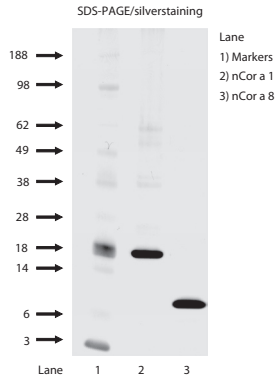
†allergy: none=no allergy; OAS=oral allergy; object=objective reaction to hazelnut

‡age in [years]

§sensitization in [IU/ml]; neg:≤0.35 IU/ml.

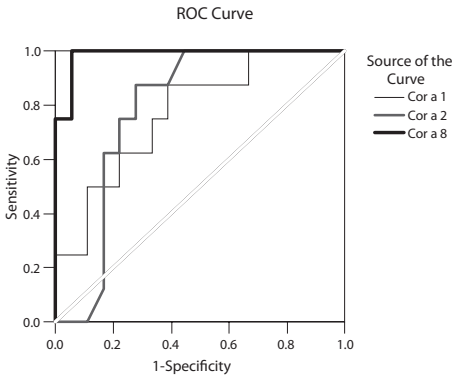
At DBPCFC						
pt	allergy†	Cor a 1§	rCor a 1§	Cor a 2§	Cor a 8§	Pru p 3§
ha 6	none	10.7	20.0	neg	neg	neg
ha 9	none	4.5	2.3	neg	neg	neg
ha 10	none	4.2	3.4	neg	neg	neg
ha 13	none	8.0	26.0	neg	neg	neg
ha 15	none	neg	neg	neg	neg	neg
ha 16	none	2.4	6.3	neg	neg	neg
ha 17	none	2.6	2.2	neg	neg	neg
ha 19	none	3.0	4.0	neg	neg	neg
ha 21	none	14.0	6.5	neg	neg	neg
ha 24	none	4.6	28.0	2.0	neg	1.3
ha 26	none	neg	neg	0.7	neg	neg
ha 29	none	0.7	neg	12.7	neg	neg
ha 33	none	neg	7.0	neg	neg	neg
ha 34	none	7.4	1.4	neg	neg	neg
ha 11	OAS	3.1	1.0	0.4	0.9	neg
ha 12	OAS	neg	neg	39.0	neg	neg
ha 22	OAS	2.9	5.0	neg	neg	neg
ha 23	OAS	0.6	neg	neg	neg	neg
ha 7	object	2.0	1.0	neg	3.7	neg
ha 8	object	3.5	0.7	neg	0.5	neg
ha 25	object	9.2	21.0	1.0	2.8	9.1
ha 27	object	7.0	2.0	0.5	2.3	0.6
ha 28	object	8.6	neg	0.7	23.3	neg
ha 30	object	15.5	17.0	2.0	9.9	neg
ha 31	object	15.3	neg	1.5	12.1	neg
ha 35	object	4.2	neg	0.8	0.8	neg
		P=0.030	P=0.567	P=0.022	P<0.001	P=0.331

Figure 4.1



Silverstaining of purified nCor a 1 and nCor a 8.

Figure 4.2



ROC curves for nCor a 8, nCor a 2 and nCor a 1 determined for the group that underwent DBPCFC with hazelnut. The group with no reaction and OAS were regarded as negative, the group with objective reactions positive.

pure by SDS-PAGE and silver-staining (fig.1). Table I lists the RAST results for these allergens, and for nBet v 1, a recombinant version of Cor a 1 (rCor a 1), nCor a 2 and peach LTP (rPru p 3) and the previously reported CAP data for specific IgE against hazelnut and birch pollen extract. [11] Levels of hazelnut-specific IgE were significantly higher ($p<0.001$) for children with objective reactions (median 32.5 kU/L) compared to children without objective reactions (median 1.5 kU/L). All eight children with objective symptoms were sensitized to nCor a 8 (median 3.25 IU/ml; range: 0.51 – 23.3 IU/ml). Two of them were also sensitized to peach LTP (rPru p 3). The ingestion of peach did not cause allergic symptoms in one child, the other never ingested peach. Only one child in the group without objective reactions was sensitized to nCor a 8 (0.9 IU/ml). This child refused to complete the challenge after developing OAS on 3 subsequent portions. Three children with objective symptoms demonstrated significant levels of specific IgE against nCor a 1 but were negative for rCor a 1 and for nBet v 1. Sensitization to birch pollen, nBet v 1 and rCor a 1 was similar in both groups ($p >0.5$).

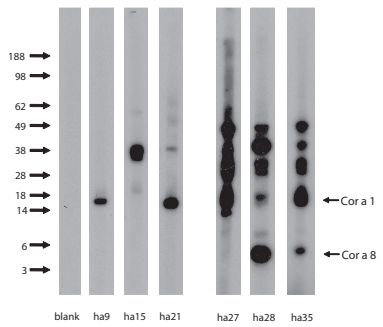
Using ROC curves (fig. 2); cut-off values for the individual allergens were calculated: 0.65 IU/ml (nCor a 8), 0.45 IU/ml (nCor a 2), 0.65 IU/ml (nCor a 1), 0.35 IU/ml (rCor a 1), 0.35 IU/ml (nBet v 1) and 0.65 IU/ml (rPru p 3). In a univariate analysis, only sensitization to nCor a 8 ($p<0.001$) and nCor a 2 ($p=0.026$) were associated with objective reactions to hazelnut during DBPCFC. Combining sensitization to nCor a 8 and to nCor a 2 in a multivariate analysis using a logistic regression model, revealed that only sensitization to nCor a 8 was independently associated to the appearance of

objective reactions in the DBPCFC (undefined OR, $p<0.001$). The probability of having an objective reaction during DBPCFC with hazelnut was 87.5% when the IgE titers to Cor a 8 were ≥0.65 IU/ml, dropping to 5.5% for IgE titers < 0.65 IU/ml. So, sensitization to nCor a 8 is the only significant and strong risk factor for objective reactions to hazelnut during DBPCFC.

Complexity of IgE reactivity to hazelnut is particularly observed in objective reactions

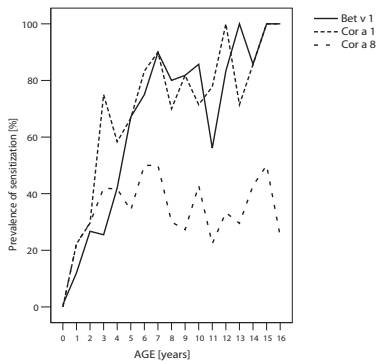
Using crude hazelnut extract on immunoblots, 7 IgE binding proteins were identified between 5 and 50 kDa (at ~ 8, 14, 17, 20, 25, 40 and 48 kDa; fig. 3). Children with objective reactions to hazelnut recognized significantly more hazelnut proteins than children without objective reactions (median 6 versus median 2.5; $p=0.001$). Five of the 8 children with objective reactions recognized a protein at ~8 kDa, corresponding to the expected size of LTP. Immunoblots with purified nCor a 8 were positive for the same 5 children (not shown).

Figure 4.3



Immunoblot with hazelnut extract for children who underwent DBPCFC with hazelnut. The numbers under each lane correspond with the numbers of the children in Table I. The first lane represents a negative control (non-atopic), then 3 children without objective reactions to hazelnut. The last 3 lanes represent children with objective reactions.

Figure 4.4



Prevalence of sensitization to nBet v 1, nCor a 1 and nCor a 8 in different age groups (0.5 – 16.6 years; median 4.5).

Sensitization to hazelnut LTP is common for hazelnut-sensitized children in a birch endemic area

To assess whether sensitization to hazelnut LTP (Cor a 8) is common among hazelnut-sensitized children in The Netherlands, 191 sera of children with a positive CAP for hazelnut were evaluated for sensitization to nCor a 8. In addition, IgE against nCor a 1 and nBet v 1 was measured. Specific IgE against nCor a 8 was present in 30.9% of the children (median: 1.4 IU/ml; range: 0.4 – 32.6 IU/ml). As expected for a birch-endemic area, prevalence of sensitization to nCor a 1 and nBet v 1

was high: 59.7% was positive for nCor a 1 (median: 3.1 IU/ml; range: 0.4 – 29.5 IU/ml) and 52.3% for nBet v 1 (median: 27.3 IU/ml; range: 0.5 - > 100 IU/ml). Fifty-seven of all children (32%) were neither sensitized to nBet v 1, nCor a 1 nor nCor a 8. These children were significantly younger (mean age 2.9 versus 7.7 years; $p < 0.001$) and demonstrated lower anti-hazelnut IgE titers (1.6 versus 2.8 IU/ml; $p < 0.001$).

All challenged children were ≥ 3.6 years of age. In the group of sensitized children more than half (105/191) were ≥ 3.6 years of age with a median age not significantly different from the challenged children (8.9 versus 7.0 years). The median level of hazelnut-specific IgE of these older children (≥ 3.6 years) was not significantly different from that of the challenged children (2.35 kU/L versus 3.95 kU/L; p=0.137). Prevalence of sensitization to Cor a 8, Cor a 1 and Bet v 1 were similar for these age-matched groups (table II), thus supporting that the challenged group was representative for the larger group of hazelnut-sensitized children.

Table II. Number and percentages of sensitization to nCor a 8, nCor a 1 and nBet v 1 for hazelnut-sensitized children older than 3.6 years compared to the challenged group (≥ 3.6 years).

	Unchallenged group ≥ 3.6 years			Challenged group ≥ 3.6 years		
	Cor a 8	Cor a 1	Bet v 1	Cor a 8	Cor a 1	Bet v 1
Tested samples (n)	107	107	102	26	26	26
Positive samples (n)	41	88	80	9	22	19
Percentage	38%	82%	78%	35%	85%	73%

Sensitization patterns to Cor a 8, Cor a 1 and Bet v 1 at different ages

The prevalence of sensitization to nCor a 8 increased up to the age of 3 years to a level of approximately 30%, around which it fluctuated in older children (fig. 4). Nineteen of the 59 Cor a 8-sensitized children (32%) were not sensitized to Bet v 1. This group was significantly younger than the other nCor a 8-sensitized children ($p<0.001$), although hazelnut and nCor a 8 specific IgE levels were similar. The prevalence of sensitization to nBet v 1 was 9% at the age two years and 67% at six years and also in older children (fig. 4). For nCor a 1 a similar pattern was observed of 16% and 67% respectively. The levels of specific IgE to both nBet v 1 and nCor a 1 were positively correlated with

age (r=0.598 and r=0.630 respectively; p<0.001).Twenty-two children had specific IgE antibodies to nCor a 1 without detectable sensitization to nBet v 1. These children were significantly younger than those with sensitization to both nCor a 1 and nBet v 1 (median 3.1 versus 8.6 years; p<0.001). Fourteen children were sensitized to both nCor a 8 and nCor a 1 without detectable sensitization to nBet v 1.

Discussion

Sensitization to hazelnut LTP (Cor a 8) was previously reported as a risk factor for more serious reactions for patients from the Mediterranean area. [4] Similar findings were reported for apple LTP. [5] In the present study it was shown that sensitization to hazelnut LTP is a significant risk factor in children from a birch-endemic area as well, where hazelnut allergy is usually associated with birch pollen sensitization and mild oral symptoms. [1-3] Of the entire hazelnut-sensitized pediatric population visiting our hospital over a period of 18 months, 30.9% was sensitized to Cor a 8.
Children with challenge-induced objective reactions were consistently sensitized to Cor a 8, whereas none of the children was sensitized who had completed the DBPCFC to the highest dose (open challenge with 10 hazelnuts) without developing objective symptoms. One child sensitized to Cor a 8 reported only subjective symptoms but refused to finish the challenge up to the highest dose. Cor a 8 sensitization detected by RAST corresponded in 5/8 cases with immunoblots with whole hazelnut and with nCor a 8. Three RAST-positive sera did not bind to Cor a 8 on immunoblot, both purified and in extract. The (partial) disturbance of the three-dimensional structure of Cor a 8 by reduction of disulphide bridges during the immunoblotting procedure may explain this discrepancy. From our results we can conclude that sensitization to nCor a 8, in particular $\geq$ 0.65 IU/ml, is a strong risk factor for objective reactions. In the statistical analyses children with OAS and those with a negative DBPCFC were taken together and compared to those with objective symptoms. At first sight this may seem unlogical from a clinical perspective. Our hypothesis was that IgE against one or more individual allergens was a risk factor for objective symptoms. IgE recognition of individual allergens was lowest in the group with a negative challenge (3/14 for Cor a 2 and 0/14 for Cor a 8), intermediate for those with OAS (2/4 and 1/4, respectively) and highest for those with objective symptoms (6/8 and 8/8, respectively). So, pooling both groups is justified because it meant that statistical analysis was performed under unfavorable condition with respect to the hypothesis that sensitization to certain individual allergens is a risk factor for the onset of objective symptoms during DBPCFC. Besides sensitization to Cor a 8, children with challenge-induced objective reactions recognized

more allergens on hazelnut immunoblot and had significantly higher levels of specific IgE to hazelnut than those without objective symptoms. Apart from Cor a 8 these included allergens of higher molecular mass, possibly Cor a 9 and/or Cor a 11, the 11S and 7S globulins of hazelnut, respectively. [22;23] Similar findings have been described for peanut, where higher levels of peanut specific IgE and more severe allergic symptoms were correlated with recognition of more allergens in peanut. [24] Interestingly, children from our study with objective reactions were also more likely to be sensitized to other nuts and peanut.[11] Possibly, cross-reactive IgE responses against 7S and 11S globulins are at the basis of the observed poly-sensitization to nuts and peanut. Purification of Cor a 9 and Cor a 11 is currently in progress to investigate this potential cross-reactivity.

Cross-reactivity between sensitization to hazelnut and birch pollen is commonly observed in individuals who live in an environment with birches.[1-3] As was expected, the prevalence of sensitization to birch pollen and its major allergen, Bet v 1 was lower at 1 year than at 6 years of age. [25;26] This was associated with sensitization to the cross-reactive hazelnut allergen Cor a 1. However, particularly younger children with Cor a 8-specific IgE antibodies were not always sensitized to Bet v 1. This suggests early sensitization to LTP (prior to and) independent from pollen sensitization. Interestingly, our study has also demonstrated that sensitization to Cor a 1 occurred independently from sensitization to Bet v 1. These children were negative to rCor a 1, indicating that the relevant isoform in the affinity-purified natural Cor a 1 was a different isoform than Cor a 1.0401. Sensitization to hazelnut Cor a 1 is generally accepted to be a cross-reactive phenomenon following primary sensitization to Bet v 1. [1] Our data are contradictory to a role of birch pollen as primary sensitizer, at least for some children, and possibly point towards primary sensitization to hazelnut Cor a 8 in conjunction with sensitization to Cor a 1. The majority of Cor a 8-sensitized children (14/19) without Bet v 1 sensitization was sensitized to Cor a 1. How this occurred remains an open question, because for most children challenged with hazelnut, the DBPCFC was the first known exposure to hazelnut. Reactions upon first exposure have been described earlier and several explanations have been proposed, ranging from unknown oral exposure (chocolate products) and cross-reactivity to another food, to sensitization in utero, via breast feeding or via skin exposure. For Mediterranean LTP allergic patients, peach has been implicated as the primary sensitizer. [27] For our group of children this is highly unlikely, because only 2/9 Cor a 8-sensitized children had IgE antibodies against peach LTP (Pru p 3). Moreover, most of these children had eaten peach without developing allergic symptoms. Of course alternative explanations can be proposed, like primary sensitization to hazel pollen Cor a 1, resulting in cross-reactivity to nut Cor a 1. Longitudinal cohort studies are needed to firmly establish this sequence of events.

Conclusion

Sensitization to Cor a 8 in children is a risk factor for severe (objective) symptoms in response to ingestion of hazelnut, not only in the Mediterranean area but also in birch-endemic areas. Sensitization to Cor a 8, but also to Cor a 1, was not always accompanied by birch pollen sensitization, especially in younger children.

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The determination of no-observed-adverse-effect levels and eliciting doses in a representative group of peanut-sensitized children

Abstract

Background: Current labeling practices for allergenic foods like peanut can be inadequate. For future regulatory and industry guidelines, information on no-observed-adverse-effect levels (NOAEL) and eliciting doses (ED) for allergenic foods is necessary.

Objective: To determine NOAEL and ED in a representative group of peanut-sensitized children, relate these data to history and sensitization, and evaluate the outcome of dietary management.

Methods: From an overall eligible group of 96 peanut-sensitized children, a representative group of 27 was evaluated by questionnaires, skin prick test, determination of specific IgE and double-blind placebo-controlled food challenge (DBPCFC) with peanut according to the international consensus protocol, with 9 doses ranging from 10 µg to 3 g peanut flour. Dietary management was evaluated over a 12-month period.

Results: Twenty-two children (81%) had a positive DBPCFC. The NOAEL in this group was 1 mg peanut flour, corresponding to 2 mg whole peanut. The ED for subjective symptoms (10 mg – 3 g) were significantly lower than for objective symptoms (100 mg – 3 g) (p=0.002). Severe reactions occurred only at high doses. ED were not correlated to previous reactions by history, SPT or specific IgE levels. All patients with a positive DBPCFC were advised to follow a strict diet. During the follow-up period, ten patients had a less strict diet likely containing traces of peanut. In 3 cases a mild reaction occurred with food products labeled 'may contain peanut'.

Conclusion: The NOAEL in a representative group of peanut-allergic children was 2 mg. Dietary compliance in half of this group was inadequate.

Introduction

In recent years peanut allergy seems to be on the rise in westernized countries, with a prevalence of 1-1.5%.[1-3] Peanuts, together with tree nuts, account for the majority of food allergic reactions with fatal outcome both in children and in adults.[4] Since spontaneous resolution of peanut allergy in children is rare[5-7]and curative treatment is not yet available,[8] the majority of children has to cope with a lifelong elimination diet. Strict elimination of peanuts is the rule because traces of peanut can cause a severe allergic reaction in sensitive subjects.[9-11] However, strict elimination is difficult, especially since food-labeling practice can be still inadequate.[12, 13] Recent developments intending to improve regulatory aspects of food labeling have led to increased 'advisory' labeling.[14, 15] This is beneficial with respect to the prevention of adverse reactions, but may in the same time lead to a rise in so-called 'may contain' labeling of products that do not necessarily contain allergenic food residues.[16] To address this problem, information on no-observed-adverse-effect levels (NOAELS) of peanut is needed. Recently, a consensus protocol for DBPCFC aimed to determine eliciting doses (ED) has been developed.[17] This protocol also facilitates comparison of results from different centers.
This study was designed to determine a NOAEL and individual eliciting doses (ED) in a representative group of peanut-sensitized children using the new consensus protocol and to investigate relating patient factors, like history and sensitization. In addition, we evaluated the outcome of dietary management post gold standard diagnostic test during a 12-month follow-up period on the occurrence of allergic reactions in everyday life.

Methods

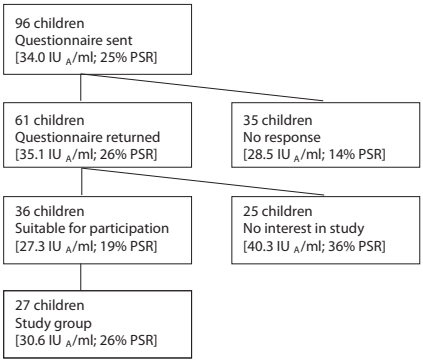
Patients

Between January 2001 and January 2003 572 children visiting the outpatient clinic of the Department of Pediatric Dermatology and Allergology were routinely screened for peanut sensitization, which was confirmed in 267 children. Peanut elimination diets were initiated in children with a history of allergic reactions to peanut and in children without any known intake of peanut. Children older than 3.5 years with sensitization to peanut and a peanut elimination diet were considered eligible. All eligible children (n=96; 67 male and 29 female; mean age 7.5 years) were approached using a standardized questionnaire about previous allergic reactions to peanut. Parents of 61 children (61/96) responded, of whom 36/61 were interested in participating and 25/61 were not interested. From the 36 interested children, 27 were included in this study to evaluate peanut allergy by

specific IgE measurement, skin prick test and DBPCFC. Parents of 35/96 children did not respond. Information on previous reactions to peanut in this group was obtained by phone using the same questionnaire. Six children were lost to follow-up.

Parents of the selected study group (n=27) completed an extensive questionnaire about atopy, elimination diet, and previous allergic reactions to peanut. SPT and specific IgE measurements were repeated and a DBPCFC was performed using the new standardized protocol[17] to establish ED. Dietary management and allergic reactions to peanut were documented during a 12-month follow-up period. All parents gave written informed consent before enrolment in the study. The study was reviewed and approved by the Central Committee of Human based Research in the Netherlands (CCMO, The Hague, the Netherlands).

Figure 5.1



Flow chart of patient selection, with specific IgE to peanut and the percentage of previous severe reactions to peanut (PSR) for each group.

Sensitization

Skin prick tests were performed on the patient's back with a standardized prick needle using commercial peanut extract (ALK-ABELLÓ, Nieuwegein, the Netherlands). Prior to the skin tests, patients discontinued antihistamines for at least 2 days. 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). The wheal reaction was measured after 15 minutes and transferred with transparent adhesive tape onto a record sheet. The area of the skin wheal was determined by computer scanning.[18] SPT responses were standardized by dividing the wheal area of the peanut prick by that obtained for the histamine control.

Specific IgE levels were determined using CAP system FEIA (Pharmacia Diagnostics, Uppsala,

Sweden) according to the manufacturer instructions. IgE levels of more than 0.35 IU_A/ml were considered positive. Besides peanut, coexisting sensitization for other allergens was investigated by CAP system FEIA. The following allergens were tested: house dust mite, grass pollen, hen's egg, birch pollen, soy, and cow's milk.

DBPCFC

Eliciting doses for peanut were investigated by DBPCFC according to the guidelines of a recently published international protocol[17] with some modifications. Exclusion criteria involved the use of β–blocking agents, ACE-inhibitors or immunosuppressive agents and any relevant disease; none of the children in this study was excluded. One medical doctor supervised all challenges in a clinical setting equipped for resuscitation and monitoring of vital signs. Prior to the test, children discontinued antihistamines (48 hours), β-mimetics (12 hours) and topical steroids (24 hours). Before starting the DBPCFC, a thorough clinical examination was performed. Blood pressure, heart rate, breath sounds, abdominal palpation and inspection of the skin using SCORAD[19] were all recorded. Challenge materials were prepared by the hospital pharmacy, consisting of 9 portions of defatted light roasted peanut flour: 10 µg, 100 µg, 500 µg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g, 3 g (protein content 50%, fat 12% by Leco 2000 method; kindly provided by the Food Allergy Research and Resource Program, University of Nebraska, USA). Portions of 10 µg to 10 mg, and of 100 mg to 3 g peanut flour were masked in 100 mg or 10 g whole-wheat instant cereal, respectively (a pinch of cinnamon powder was added to improve masking). Four placebos were randomly interspersed and consisted of whole-wheat instant cereal and a pinch of cinnamon. The use of interspersed placebos was chosen because the procedure could be completed in one day. Just before administering, the test meals were mixed with 1 tablespoon applesauce (in case of the low doses: 10 µg to 10 mg peanut flour) and with 10 ml warm water with 5 tablespoons applesauce (in case of the higher doses: 100 mg to 3 g peanut flour). The doses were given in time intervals of 15 minutes for the low doses and 30 minutes for the higher doses. The interval of 15 minutes (a slight modification from the protocol) was chosen for the low doses because of practical reasons in order to attain full cooperation of the child. In case of a reaction, the next dose was postponed until all symptoms were resolved. The challenge was discontinued when objective symptoms occurred or when consistent subjective symptoms occurred on at least 3 subsequent doses. The symptoms were treated with appropriate medication.[20] Reactions like the oral allergy syndrome (OAS),[21] nausea and abdominal pain were referred to as subjective symptoms. Objective symptoms indicative of an allergic reaction included urticaria, facial swelling, rhinoconjunctivitis, vomiting, diarrhea, dyspnea, bronchoconstriction,

and tachycardia. Symptoms were graded according to the scale of Mueller from 0-4. [22] Children with a reaction to peanut stayed in the ward for at least 4 hours after disappearance of the allergic symptoms. If no reaction occurred during the DBPCFC, the challenge was continued with an open dose of 10 g peanuts. The peanuts were fried under controlled circumstances to exclude cross-contamination with nuts. Allergenicity appeared comparable with that of roasted peanuts (data not shown). These children were kept under medical supervision for at least 2 hours after the last ingested dose. To document possible late reactions, all parents were contacted by phone the day after the challenge.

Follow-up

At the end of each DBPCFC, dietary advice was given to the parents. After a negative DBPCFC, children were allowed to eat peanut. After a positive DBPCFC, dietary advice remained to avoid peanut. Children with severe reactions during DBPCFC (stridor, bronchoconstriction, and tachycardia) were advised to use a specified list of foods to achieve strict avoidance. This list indexes all food products and brands that definitely do not contain traces of peanut (provided by the Netherlands Nutrition Center, The Hague, the Netherlands). Food products are only listed when they are produced in peanut-free factories and use non-contaminated raw materials. Each year this list is updated with information provided by collaborating factories. All parents from children with a positive DBPCFC were informed that not-listed food products, and especially food products with 'may contain peanut' labels, carry a certain risk of containing substantial amounts of peanut. All parents were asked to carefully document the ingestion of food products and describe any allergic reaction in a diary. This diary was compiled with help of a specialized dietician. After 3, 6 and 12 months the parents of children were contacted and dietary management and allergic reactions were evaluated with help of interviews and the diary.

Statistics

Values were reported as the mean, unless otherwise indicated. SPT, specific IgE and ED were not normally divided, even after logarithmic transformation; hence all calculations were done with non-parametric tests. The relevance of placebo reactions was evaluated using an adaptation of the Briggs' model,[23] by categorizing the data in non-responders (n=5), active responders (n=19), placebo responders (n=0) and combined placebo and active responders (n=3). The Mann Whitney U test was used to calculate differences in SPT, specific IgE and ED between groups. The χ^2 test with Fisher's correction was performed between the proportions of previous reactions between negative

and positive DBPCFC. The Wilcoxon test was used to calculate paired differences in ED between subjective and objective symptoms. Spearman's Rho was calculated as correlation factor between eliciting doses, severity of reaction, SPT and specific IgE outcomes. Differences were considered significant if the p-value was ≤ 0.05. Statistical analysis was performed using the Statistical Program SPSS (version 12.0, SPSS Inc., 2001, Chicago USA).

Table I. Characteristics of study group (n=27).

Characteristic	Number of children (%)
Sex	
Male	18 (67%)
Female	9 (33%)
Mean age	7.2 years
Atopic history	
Other food allergies	24 (89%)
Atopic dermatitis	18 (67%)
Allergic asthma	15 (56%)
Seasonal rhinoconjunctivitis	11 (41%)
Sensitization (elevated specific IgE)	
Peanut	23 (85%)
House dust mite	21 (78%)
Grass pollen	19 (70%)
Hen's egg	18 (67%)
Birch pollen	17 (63%)
Soy	15 (56%)
Cow's milk	10 (37%)
Previous reactions to peanut	
At least one	17 (63%)
None	10 (37%)

Results

Patient characteristics

A group of 27 children (18 male and 9 female) participated in this study for complete evaluation of peanut allergy (Table I). The average age was 7.2 years (range 3.7-15 years). To investigate whether this study group (n=27) was a representative selection of the original peanut-sensitized population (n=96), peanut-specific IgE levels and the percentages of previous severe reactions to peanut (dyspnea, bronchoconstriction, shock) were compared (Fig. 1). Compared to the original population of peanut-sensitized children (n=96), the study group had comparable specific IgE levels (30.6

versus 34.0 IU_A/ml) and the percentage of previous severe reactions to peanut was similar (26% versus 25%). The study group had lower specific IgE levels (30.6 versus 40.3 IU_A/ml) and a lower percentage of previous severe reactions to peanut, dyspnea, bronchoconstriction and/or shock (26% versus 36%), than the children that were not interested (n=25). These differences were not statistically significant.

In the study group, other food allergies were present in 89%, atopic dermatitis was present in 67%, allergic asthma in 56%, and seasonal rhinoconjunctivitis in 41% of the children. Before the DBPCFC procedure, SPT and specific IgE levels were re-evaluated (Table II). SPT results varied from 0 to 23.9 mm^2 peanut / mm^2 histamine (mean 6.9). IgE levels ranged from <0.35 IU_A/ml to >100 IU_A/ml (mean 36.9 IU_A/ml). At this point, one child lost the sensitization to peanut. The majority of patients was also sensitized to common food and inhalant allergens. Seventeen children (63%) reported a previous allergic reaction to peanut during the peanut elimination period (a half to ten years before the DBPCFC). In this group, 4/17 children had a reaction after skin contact, resulting in contact urticaria, associated with facial swelling in 2 children. Thirteen children (13/17) developed symptoms after ingestion: OAS (n=11), urticaria, facial swelling and/or rhinoconjunctivitis (n=12), abdominal pain, vomiting and/or diarrhea (n=5), dyspnea and/or bronchoconstriction (n=7). One child had to be admitted to the hospital for severe dyspnea. These allergic reactions to peanut usually occurred after the inadvertent ingestion of peanut, peanut butter, peanut sauce or pastries clearly containing peanut. Four children had eaten food products containing hidden peanut: a vegetarian meatball, chocolate ice cream, a chocolate candy bar or a chocolate lollypop.

Factors related to positive DBPCFC

DBPCFC was positive in 81% of the children (22/27) confirming the diagnosis of peanut allergy. A positive DBPCFC was associated with previous reactions to peanut by history. Of the children without a previous reaction by history, 60% (6/10) had a positive DBPCFC, whereas 94% (16/17) of the children with a previous reaction had a positive DBPCFC (p=0.047). In comparison, 73% of children with a positive DBPCFC had a previous reaction to peanut, compared to 20% of children with a negative DBPCFC. In our study, sensitization to peanut in children with a positive DBPCFC was significantly higher than in children with a negative DBPCFC, determined both by SPT (p=0.002) and by specific IgE (p=0.010). Cut-off levels for SPT wheals or specific IgE could not be calculated because the number of patients with a negative DBPCFC was too small (n=5).

Table II. Sensitization, symptoms by history and the results of DBPCFC for children without (n=10) and with previous reactions to peanut (n=17).

	Sensitization		History		DBPCFC		
No	SPT*	IgE†	Symptoms‡	Symptoms‡	NOAEL§	ED subjective§	ED objective§
pi 4	0	0	no previous reaction	**none**	-	-	-
pi 6	1	0	no previous reaction	**none**	-	-	-
pi 7	1	0	no previous reaction	**none**	-	-	-
pi 18	2	0	no previous reaction	**none**	-	-	-
pi 25	26	1.0	no previous reaction	oas, fs, ap	300	1000	>3000
pi 9	7	6.9	no previous reaction	rc, vom	1000	-	3000
pi 26	21	48	no previous reaction	gu, rc, vom	1000	-	3000
pi 27	1	7.5	no previous reaction	oas, fs, rc, ap, dysp	300	1000	3000
pi 20	8	4	no previous reaction	oas, rc, ap, dia, dysp	1000	3000	>3000
pi 19	10	100	no previous reaction	rc, vom, bro	300	-	1000
pi 12	0.2	55	cu	**none**	-	-	-
pi 14	1	0.42	oas, fs, dysp	oas	3000	>3000	-
pi 41	3	35	oas, urt, fs, rc	oas, cu, fs	1000	3000	>3000
pi 36	3	100	oas, fs, vom, dysp	oas, ap	1	10	-
pi 15	3	3.0	oas, vom, dysp	oas, rc, ap	3000	>3000	>3000
pi 37	9	3.3	oas, fs, rc, vom, dysp	oas, fs, urt, rc, ap	10	100	>3000
pi 17	6	100	cu, fs	oas, vom	1	10	300
pi 40	3	3.3	oas, urt, vom	nau, vom	300	1000	>3000
pi 29	2	15	cu	oas, ap, vom	300	1000	3000
pi 16	1	36	oas, fs, bro	oas, rc, vom	1000	3000	3000
pi 21	6	21	oas, urt	oas, rc, ap, vom	100	300	1000
pi 22	4	61	oas, fs, rc, dia, bro	oas, ap, vom, dia	100	300	>1000
pi 38	10	93	gu, fs	gu, rc, ap, vom, dia	3000	>3000	>3000
pi 39	13	2.5	urt, rc	oas, vom, rc, dysp	100	300	>300
pi 11	10	100	cu, fs	rc, dysp	10	-	100
pi 24	24	100	oas, gu, fs	gu, vom, str	300	-	1000
pi 28	10	99	oas, fs, rc, bro	oas, gu, rc, bro, tach	300	1000	3000

* skin prick test (SPT) in area peanut / area histamine
† IgE in IU_A/ml
‡ symptoms: oas=oral allergy syndrome, cu=contact urticaria, urt=urticaria, gu=generalized urticaria, fs=facial swelling, c=conjunctivitis, rc=rhinoconjunctivitis, ap=abdominal pain, vom=vomiting, dia=diarrhea, dysp=dyspnea, str=stridor, bro=bronchoconstriction, tach=tachycardia
§ NOAEL (no-observed-adverse-effect level) and ED (eliciting dose) in mg

Symptoms during DBPCFC

Symptoms during DBPCFC varied in severity from mild (OAS, several urticaria, abdominal pain) to severe (bronchoconstriction, tachycardia). Table II describes the reactions during DBPCFC, as well as previous reactions and sensitization for each patient, displayed in order of severity of symptoms during DBPCFC. Usually, symptoms developed within 30 minutes after ingestion. Generalized

urticaria developed later in the course of an allergic reaction, usually 50-90 minutes after the last ingested dose.

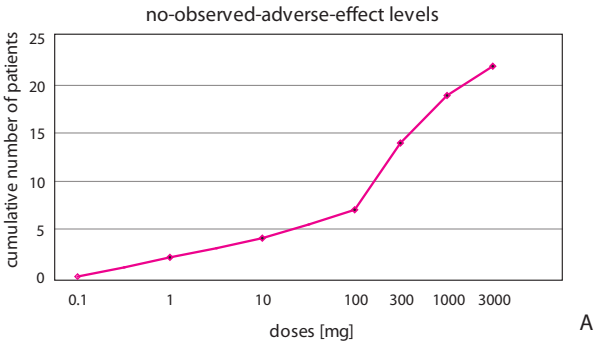
Late reactions like deterioration of atopic dermatitis were not observed in this study. Severe reactions during DBPCFC involving the respiratory tract and/or the circulatory system were seen in 3/22 (14%) children, but only at high dose. The first child developed vomiting and rhinoconjunctivitis, followed by severe bronchoconstriction after ingestion of 1 g peanut flour. The second child vomited after 1 g, started coughing after an hour and subsequently developed stridor and generalized urticaria. The third child complained of OAS after the ingestion of 3 g peanut flour, immediately followed by acute dyspnea due to bronchoconstriction. Systemic administration of antihistamines and adrenaline, and inhalation of steroids and bronchodilators, led to a temporary improvement. After 15 minutes generalized urticaria, severe dyspnea and tachycardia developed, needing a second treatment course. Another 2 children with symptoms like vomiting and rhinoconjunctivitis developed generalized urticaria that worsened despite intravenous antihistamines and steroids, but responded well after additional administration of adrenaline. Overall, 20/22 children developed objective symptoms during DBPCFC, whereas 2/22 exclusively reported subjective symptoms. Subjective symptoms were reported after 7% (5/74) of the placebo portions by 3 children. Subsequently, all 3 patients developed objective reactions to active portions. Placebo reactions had no impact on the final results using an adaptation of the Briggs'model.[23] Determination of the NOAEL was also not influenced by the placebo reactions.

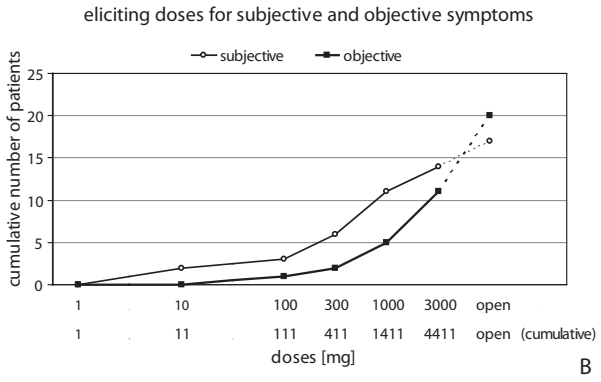
To determine if the severity of the reaction during DBPCFC was related to sensitization, symptoms were graded according to the scale of Mueller from 0-4.[22] The severity of the reaction during DBPCFC was weakly correlated to SPT reactivity (rho=0.49; p=0.021), but not to specific IgE levels or to the severity of the reaction by history.

NOAEL and ED for subjective and objective symptoms as determined by DBPCFC

All children tolerated a dose of 1 mg peanut flour. Hence, the NOAEL in our study was 1 mg peanut flour, which corresponds to 2 mg peanut (Fig 2A). The lowest ED was 10 mg (n=2), causing OAS. The ED for subjective symptoms ranged from 10 mg to 3 g and was significantly lower than that for objective symptoms that ranged from 100 mg to 3 g (p=0.002, Fig 2B). Subjective symptoms were OAS, nausea and/or abdominal pain. Objective symptoms consisted of several urticaria, generalized urticaria, facial swelling, rhinoconjunctivitis, vomiting, diarrhea, hoarseness, stridor, bronchoconstriction, and/or tachycardia. In 2/22 reacting children, no objective signs were observed. Five (5/22) children developed objective symptoms without subjective symptoms. In

Figure 5.2





A) Distribution of NOAEL
B) Distribution of ED for subjective (orange) and objective symptoms.

12/22 cases subjective symptoms preceded objective symptoms. In these children, ED for subjective symptoms were ≥ 10 mg, and for objective symptoms ≥ 300 mg. In 3/22 children ED for subjective and objective reactions were similar (≥ 3 g). In children without a previous reaction to peanut, the ED was ≥ 1000 mg and in the group with previous reactions ≥ 10 mg. Children with previous reactions (by history) to hidden traces of peanut (n=4) had a low ED of 10 mg, 100 mg, and 300 mg (n=2).

With respect to SPT or specific IgE levels, a correlation with ED was not present. The severity of the reaction by history or during DBPCFC, as graded by Mueller, [21] was not correlated to the ED either.

Dietary management during a 12-month follow-up period

Dietary restrictions for peanut were no longer necessary for the 5 children with a negative DBPCFC (Table III), and they began the introduction of peanut into their diet. Twenty-two children had a

Table III. Accidental allergic reactions during a 12-month follow-up period in 3 different dietary management groups.

		DIETARY MANAGEMENT		
		no restriction (n=5)	less strict (n=11)	strict (n=11)
ACCIDENTAL ALLERGIC REACTIONS	to 'may contain peanut' products	0	3	0
	to other products containing peanut	0	3	0
	number of patients	0	4	0

positive DBPCFC. After DBPCFC, 11 children (11/22) had a very strict dietary management which was achieved by using a specified list indexing all food products and brands that do not contain traces of peanut. The other group (11/22) had a less strict peanut free diet and did not use the specified list. The parents of 10 children (10/11) indicated that they occasionally used food products that might contain traces of peanut.

All three groups were followed for 12 months. During the 12-month follow-up period, all of the children with negative DBPCFC ingested peanuts without problem. The children that followed the strict diet using specified lists of peanut free products did not experience any allergic reaction to peanut. In the group that was less strict, mild OAS was reported by 3 children after the ingestion of foods with a 'may contain peanut' label. Due to accidental ingestion, 3 children from this same group experienced a mild to moderate allergic reaction. Two children developed a reaction, consisting of vomiting (associated with facial swelling in one child), after the ingestion of crisps from a bowl that contained peanuts before. The third child developed hoarseness and stridor one hour after the ingestion of ice cream with peanut and chocolate crunch topping.

Discussion

The aim of this study was to determine the NOAEL and the ED for peanut in a representative group of peanut sensitized children and to relate these data to history and sensitization to peanut. Furthermore, we aimed to evaluate the outcome of dietary management during a 12-month follow-up period. In our opinion this is the first study that has determined a NOAEL in peanut-sensitized children. We used the recently published consensus protocol that is developed to determine a NOAEL and lowest ED. Most studies in children so far reported reactions to the first dose, rendering the study inappropriate to determine a NOAEL.[24-26] In our study, the NOAEL was 1 mg peanut flour,

corresponding to 2 mg peanut. This is within the limits of currently available detection systems of peanut (0.2-1.2 mg/kg).[27] The international consensus protocol was developed to set uniform guidelines about performing double blind placebo controlled food challenges. In this way, results of our study can be combined with other studies on this subject. It might be good to challenge larger numbers of patients, preferably in other centers and countries, to increase the value of the obtained data.

A major concern in most studies is that patient selection occurred based on previous reactions and sensitization, and the most sensitive patients were excluded.[24-26] One of our objectives was to study a representative group of peanut-sensitized children. The children in our study had comparable specific IgE levels and incidence of previous severe reactions as the initially contacted peanut-sensitized population (Fig 1). During DBPCFC 14% had a severe reaction. One child (4.5%) developed cardiovascular symptoms, which is comparable to the study of Morisset et al. (3%).[24] All together, these data might reflect the fact that we did not exclude the more sensitive patients and studied a representative group of children.

The lowest ED in our study group was 10 mg, resulting in subjective symptoms (OAS) and corresponding to 20 mg peanut. This is in line with data from another study determining ED for peanut in children who found a lowest ED of 5 mg.[24] In adults, ED as low as 400 µg whole peanut have been reported, which is 50-fold lower than in our study.[10] Since at these low doses only OAS was reported, adults might be more aware of these particular symptoms. Usually adults have more concomitant food allergies, especially pollen-related, that commonly cause OAS.[28] This also explains why in children more objective symptoms (91%) were reported in contrast to adults.[9, 11] The ED for objective symptoms was significantly higher, namely 100mg, corresponding to 200 mg whole peanut. This is comparable with data found in adults by Wensing et al.[11] (40 mg) and Hourihane et al.[9] (20 mg whole peanut).

Factors that may influence the ED are sensitization, the severity of the peanut allergy,[11] and the matrix in which the peanut is hidden.[29] In our study, the ED was not correlated to the level of peanut-specific IgE, SPT reactivity or the severity of peanut allergy by history. Wensing et al.[11] showed that in adults low ED during challenge was correlated to severity of previous reactions, and to a lesser extent to reactions during DBPCFC. This result could not be reproduced in children. The ED in children with previous reactions to hidden peanut were all in the low range (10 mg – 300 mg peanut flour), suggesting that the ED reflects the situation in daily life. The ED, together with other factors as sensitization and history, might be used to come to a tailor-made dietary advice. We suggest only a less strict diet to patients with a history of exclusively mild reactions, a high ED (if

measured), and no history of reactions to traces (checked by a trained dietician).

Dietary management on the occurrence of allergic symptoms in everyday life was also studied. So far data in literature are almost non-existing.[30, 31] We are aware of the unique situation in the Netherlands, which allows us to use a list that indexes products 'free from peanut'. Additionally, all patients received information on how to avoid peanut in daily life, with special attention to foods being likely unsafe. The effect of such a strict regime on the occurrence of accidental allergic reactions appeared beneficial, since no children that carefully followed the advice experienced a reaction during the 12-months follow-up period. However, 10/27 children, including one child with a moderate to severe reaction (generalized urticaria with complete loss of bowel control) during DBPCFC, admitted to be less strict with dietary management than advised. This finding regarding compliance with respect to medical advice in patients with chronic atopic diseases has been reported earlier.[32, 33] Remarkably, 3 of the 6 reactions reported during follow-up could be related to 'may contain peanut' labeled products. This is especially important with regard to the increasing use of 'may contain peanut' labeling[16], due to recent regulatory changes[14], which has a great impact on the food choices of allergic individuals and may influence the compliance to a diet in a negative way. The fact that a substantial number of patients in daily practice already follows a less strict diet underlines the importance of good instructions of patients regarding the safety of foods with special attention to foods labeled as 'may contain peanut'. Data on NOAEL will contribute to future guidelines regarding the development of safe foods.

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Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time

Abstract

Background: In peanut-allergic adults, IgE is mainly directed to Ara h1 and Ara h2. More recently, a role for Ara h6 has been suggested. In contrast to adults, IgE in children can fluctuate over time. Therefore children may have a more dynamic reactivity to peanut.

Objective: To examine the IgE reactivity to major peanut allergens in peanut-allergic children at two subsequent time points.

Methods: Twenty children (3-15 years old) with peanut allergy, confirmed by double-blind placebo-controlled food challenge (DBPCFC), were included. Just before and 20 months after DBPCFC, IgE reactivity to purified Ara h1, Ara h2, Ara h3 and Ara h6 was studied by immunoblots and skin prick tests (SPT).

Results: Before DBPCFC, all peanut-allergic children showed IgE reactivity to Ara h2; Ara h6 was recognized by 16 children, and Ara h1 and Ara h3 by 10 children. After 20 months, peanut-specific IgE levels (median 23 kU/l) and the individual recognition of major allergens were comparable to the levels and recognition before challenge (median 28.2 kU/l). SPT with Ara h2 and Ara h6 was positive in most children, whereas SPT with Ara h1 and Ara h3 was positive in approximately half of the children. Ara h6 induced the largest wheals. None of the parameters were related to the severity of peanut allergy.

Conclusion: Ara h2 and Ara h6 are the most frequently recognized major peanut allergens in children. The individual reactivity to the major peanut allergens remained stable over time, despite DBPCFC.

Introduction

Peanut allergy is among the most important food allergies in westernized countries. [1-4]

Several proteins in peanut have been implicated as allergens. Ara h1, belonging to the vicilin family, and Ara h2, a member of the conglutin family, were thought to be the most important, because these allergens are recognized by IgE in 70-90% of patients with peanut allergy. [5-9] Ara h3, a glycinin protein, is recognized by only 45% of patients with a convincing history of peanut sensitivity. [10] Ara h6 shows homology with Ara h2. In recent studies we showed that adult peanut-allergic patients recognized Ara h6 to a similar extent as Ara h2, indicating that Ara h6 should be considered a major peanut allergen as well. [11;12] Inhibition experiments demonstrated that IgE-binding to Ara h6 was cross-reactive with Ara h2. The diversity of IgE allergen binding rather than binding to one specific allergen has been related to the severity of the reaction to peanut. [13]

Most studies investigating the reactivity to major peanut allergens were conducted in adults. It is assumed that IgE of children binds to similar proteins as IgE of allergic adults. In children with presumed peanut allergy, IgE specific for Ara h1, Ara h2 and Ara h3 has been described. [5;6;8;9;14] These studies were conducted with whole peanut extract or with only purified Ara h1 and Ara h2. Moreover, the finding that Ara h6 is an important allergen has not yet been confirmed in children. We think it is important to investigate IgE reactivity in children to all these purified major allergens simultaneously, and to illustrate this with *in vivo* reactivity to purified allergens by skin prick tests (SPT) in children.

Sensitization to peanut in children may be more dynamic than in adults. In children, the level of peanut-specific IgE can fluctuate over time, independent of exposure to peanut. [15] This is in line with the occurrence of fluctuating allergic sensitization described for several other food allergens in young children. [16] No study has recorded the IgE reactivity to the major peanut allergens over time so far.

The aim of the present study was to investigate the reactivity to peanut extract as well as to purified major peanut allergens (Ara h1, Ara h2, Ara h3 and Ara h6) by immunoblots and skin prick tests (SPT) in children with peanut allergy, confirmed by double-blind placebo-controlled food challenge (DBPCFC). To analyze the dynamics of IgE reactivity over time, we reinvestigated the reactivity to the major peanut allergens 20 months after DBPCFC.

Methods

Study population

Twenty children with peanut allergy (6 female and 14 male; age 3-15 years) were recruited from the Department of Pediatric Dermatology/Allergology at the University Medical Centre Utrecht. These children participated in an earlier study to determine eliciting doses (ED) for peanut and were a representative sample from the population of peanut-sensitized children in our outpatient clinic with regard to specific IgE levels and severity of previous allergic reactions to peanut. [17] Inclusion criteria consisted of a positive DBPCFC with peanut, elevated peanut-specific IgE and positive skin prick test (SPT). Besides peanut allergy, 19 children had other food allergies, mainly nut allergy. All had a history of atopic dermatitis, 15 children of allergic asthma and 8 children of seasonal rhinoconjunctivitis. Parents of these children were asked to document the (inadvertent) ingestion of food products labelled "may contain peanut" prospectively, and describe any allergic reaction in a diary. All parents gave written informed consent before enrolment in the study. The study was reviewed and approved by the Central Committee of Human based Research in The Netherlands (CCMO, The Hague, The Netherlands).

Clinical evaluation

DBPCFC was performed as described previously. [17] In short, increasing amounts of defatted peanut flour were given with time-intervals of 15-30 minutes: 0.01, 0.1, 0.3, 0.5, 1, 10, 100, 1000 and 3000 mg. Four placebos were interspersed randomly. An open challenge of 10 peanuts was the last step in the procedure. The challenge was discontinued after the occurrence of objective symptoms. After the occurrence of subjective symptoms (oral allergy, abdominal pain and nausea), the DBPCFC was continued after the disappearance of these symptoms. For determination of the ED, the first dose to provoke symptoms, either subjective or objective, was used.

The severity of allergic reactions was divided according to Muller in mild (grade 0 and 1), moderate (grade 2) and severe (grade 3 and 4). [18] Mild reactions were classified as symptoms of the oral cavity, the skin and/or mucous membranes (urticaria, angio-oedema, rhinitis, conjunctivitis), moderate reactions included gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain), and severe reactions consisted of respiratory symptoms (bronchoconstriction, stridor, hoarseness) and/or cardiovascular symptoms.

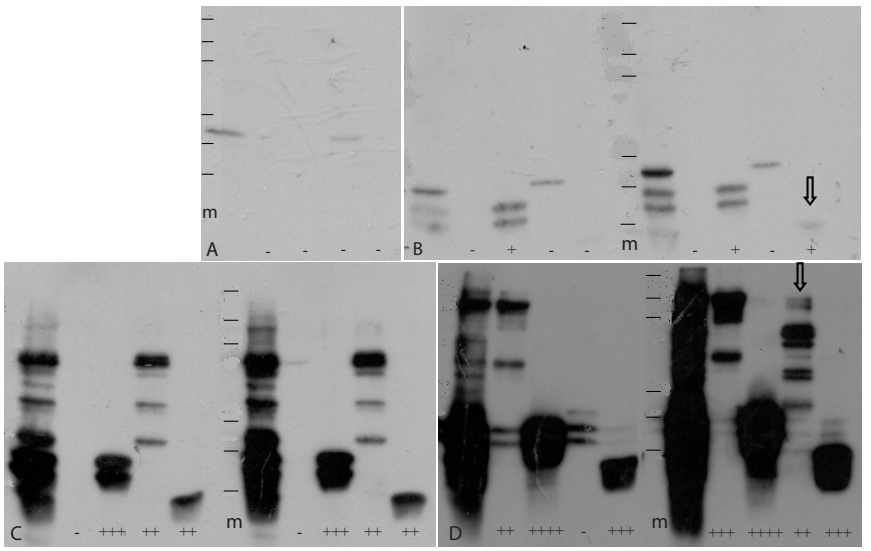
Peanut-specific IgE levels

Serum samples were collected for each patient at the day of challenge (t=0) and 20 months after (t=20). Peanut-specific IgE was determined in all samples by CAP-system FEIA according to manufacturer's instructions (Phadia, Uppsala, Sweden). IgE > 0.35 kU/l was considered positive.

Purified major allergens

Previously developed purification protocols were used for the preparation of Ara h1, [19] Ara h2, [11] Ara h3, [20] and Ara h6, [11] with purity of >95% as judged by SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining. Individual peanut allergens were sterilized and stored as described before. [7]

Figure 6.1



Examples of immunoblots: a control subject without peanut sensitization (A), peanut-allergic subjects with low peanut-specific IgE (No 17; 3.3 and 1.9 IU/ml), with moderate level of specific IgE (No 2; 15 and 23.5 IU/ml), or with high levels of specific IgE (No 5; 163 and 309 IU/ml) at t=0 and t=20 months (B-D, respectively). The extent of the IgE reactivity is described below each lane on the immunoblot. The lanes represent CPE, Ara h1, Ara h2, Ara h3 and Ara h6 from left to right. Marker lanes are indicated with "m", representing proteins at 250, 100, 50, 25, 20 and 15 kDa. Differences between t=0 and t=20 months are indicated with an arrow. Lanes of Ara h1 and Ara h 3 in panel D, t=0, show a minor reactivity at the height of Ara h 2. This is most likely due to a minute overflow of the Ara h 2 lane, occurred during loading of the gel. Impurity of the Ara h 1 and Ara h 2 is not likely based on the lack of reactivity at the height of Ara h 2 in the Ara h 1 and Ara h 3 lanes in panel D, t=20.

SDS-PAGE and immunoblot

SDS-PAGE was performed using a BioRad Mini Protean II system (BioRad, Hercules, CA, USA) with 15% acrylamide gels (15 x 10 cm) according to manufacturer's instructions. Crude peanut

extract (CPE, 2 μg) and all 4 purified major peanut allergens (0.4 μg per allergen) were loaded in separate lanes. After protein separation, the proteins were transferred to polyvinyldifluoride sheets (Immobilon-P, Millipore Corp. Bedford, MA). Membranes were blocked in 4% dried milk (Protifar Plus, Nutricia, Cuijk, the Netherlands) in PBS o/n at 4°C. Patient serum of both time points (t=0 and t=20 months) was diluted 1:50 in dilution buffer (PBS, containing 0.5% dried milk and 0.02% Tween-20) and applied on the membranes for 1 h at room temperature. Bound IgE was detected using peroxidase-conjugated goat-anti-human IgE (Kirkegaard Perry Limited, Guildford, UK) diluted 1:30.000 in dilution buffer and a subsequent staining reaction for peroxidase activity using the ECL technique (Amersham Life Science, Amersham, UK) according to the manufacturer's instructions. We scored the IgE binding from – (no visible bands) to ++++ (very broad and thick bands) by visually analyzing all blots with 3 investigators reaching consensus.

SPT with whole peanut extract and purified major allergens

SPT were performed on the patient's back or on the flexor side of the forearm with a prick needle and commercial peanut extract (ALK-Abelló, Nieuwegein, The Netherlands). Purified Ara h1, Ara h2, Ara h3 and Ara h6 were used in serial tenfold dilutions, ranging from 100 μg/ml to 1 μg/ml. The dilutions were prepared using a diluent containing 50% glycerol (v/v), 0.9 % NaCl (w/v), 0.4% phenol (w/v) and 0.3% human serum albumin (HSA) (w/v) in PBS. Histamine dihydrochloride (10 mg/ml) and the glycerol diluent of the SPT-extracts served as positive and negative controls, respectively. Because peanut induces larger wheals in children than in adults (data not shown), we started with 1 μg/ml in the first group of children out of safety precautions. The next groups were tested with higher concentrations (10 and 100 μg/ml) as no serious reactions occurred. The order in which the children were tested was random. SPT reactivity was measured after 15 minutes and transferred with transparent adhesive tape onto a record sheet. The area of the SPT wheal was determined by computer scanning. [21] SPT responses were standardized by dividing the wheal area of the peanut prick by that obtained from the histamine control. SPT ratios ≥ 0.25 were considered positive. [12]

Statistics

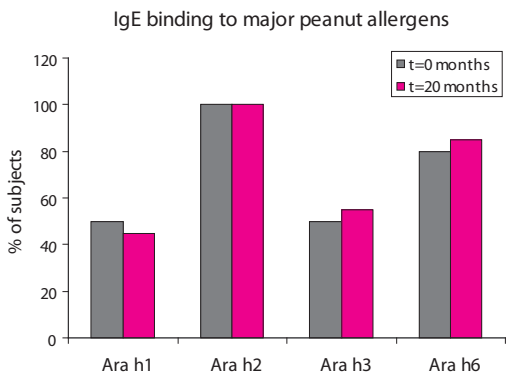
All analyses were performed with nonparametric tests (Mann-Whitney U test and Kruskal-Wallis test for comparison between groups and correlations with Spearman's rank coefficient). For quantitative analysis of the immunoblots, we labeled the values from 0 (corresponding to -) to 4 (corresponding to ++++). Calculations were performed using SPSS (version 12, SPSS Inc., 2001, Chicago, USA). P values <0.05 were considered significant.

Peanut allergy and dietary management during 20 months

The severity of the reaction and the ED determined by DBPCFC are displayed in Table I. All 20 children developed an objective reaction, ranging from mild to severe. Mild reactions were oral symptoms with abdominal pain and vomiting, whereas the most severe reaction consisted of generalized urticaria, rhinoconjunctivitis, severe bronchospasm and tachycardia._The ED per individual ranged from 10 mg to more than 3 gram.

During the 20 months after DBPCFC, all children continued to eliminate peanut from their diet. Parents of 13 children indicated to be less strict with the elimination of peanut than advised. Four children reported OAS after the ingestion of products that might contain peanut. Two children with a less strict diet experienced an objective allergic reaction to peanut within these 20 months.

Figure 6.2



IgE reactivity to major peanut allergens on immunoblot. The percentage of individuals (n=20) recognizing the specific major allergens (Ara h1, Ara h2, Ara h3 and/or Ara h6) are displayed for t=0 and t=20 months.

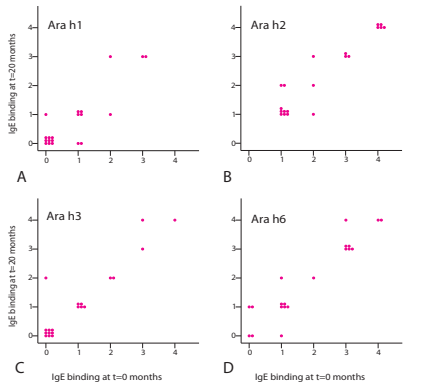
Peanut-specific IgE recognizes predominantly Ara h2 and Ara h6

Sera were collected before DBPCFC and analyzed by IgE-immunoblotting. Figure 1 shows representative examples of immunoblots. Ara h2 and Ara h6 were recognized by the majority of the children, whereas Ara h1 and Ara h3 were recognized by IgE from the serum of approximately half of the children, as shown in Figure 2. Eight children recognized all 4 major peanut allergens, 3 children recognized 3 major allergens (Ara h2 and Ara h6, together with either Ara h1 or Ara h3), 6 children recognized 2 major allergens (in 5 cases Ara h2 and Ara h6 and in 1 case Ara h1 and Ara h2), and 3 children recognized only Ara h2. The group with strict elimination showed similar

recognition of major allergens as the group who followed a less strict dietary management. The number of recognized allergens correlated with the level of peanut-specific IgE (r = 0.68; p=0.001). No correlation between the number of recognized allergens and the age of the children was found (r = -0.37; p=0.104).

The extent of IgE binding, determined by visually analyzing the bands, varied widely between the different individuals and the different allergens (Table I). The IgE binding to Ara h2 and Ara h6 was significantly more intense than the IgE binding to both Ara h1 and Ara h3 (all p<0.05). No difference in IgE binding was shown between Ara h1 and Ara h3 or between Ara h2 and Ara h6.

Figure 6.3



IgE reactivity at t=0 months compared to t=20 months for Ara h1 (A), Ara h2 (B), Ara h3 (C), Ara h6 (D). Each dot represents one individual.

Recognition of major peanut allergens by IgE remains stable 20 months after challenge

Sera were also obtained 20 months after the challenge (Table I). Peanut-specific IgE levels at that time point (range 1.0 - 616 kU/l; median 23.0 kU/l) were comparable to the levels before challenge (range 1.0 – 815 kU/l; median 28.2 kU/l; p=0.218).

At t=20 months Ara h2 was still recognized by all children (100%), Ara h6 by 17 (85%), Ara h3 by 11 (55%) and Ara h1 by 9 (45%). The recognized number and the extent of IgE binding to Ara h1, Ara h2, Ara h3 and Ara h6 on the blots at t=20 months did not differ between both time points (Figure 3). Four major allergens were investigated in each child (n=20), hence 80 tests were performed at both time points. The recognition at t=0 was comparable to t=20 months in 73/80 cases (91%). Six children showed minor changes between t=0 and t=20 months. An example of such a change is shown in Figure 1C. These changes were not related to age, level of specific IgE or dietary management during the 20 months (strict, less strict). One child (No. 15) clearly recognized

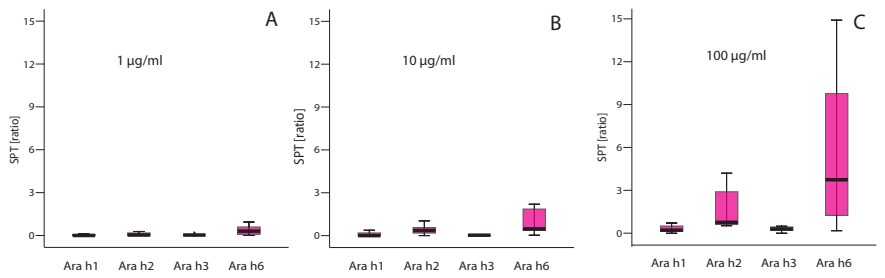
Ara h3 after 20 months (++) in contrast to t=0 (-), as is shown on the immunoblot in Figure 1D. This child had a strict elimination diet with no apparent ingestion of peanut, and reported no reactions to peanut during the observation period of 20 months.

SPT is mainly positive for Ara h2 and Ara h6

The SPT ratios at t=20 months (median 6.9; range 0.8 – 25) were not significantly different from SPT ratios at t=0 months (median 4.3, range 2.3 – 23; p=0.25).

SPT for the major peanut allergens were performed at t=20 months at concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml. There was a clear dose-response relationship for all purified allergens tested, with larger wheals at higher concentrations (Figure 4). The largest SPT reactivity was induced by Ara h6, followed by Ara h2.

Figure 6.4



SPT with major peanut allergens. The interquartile ranges of SPT ratios (area peanut allergen/area histamine) at t=20 months (Ara h1, Ara h2, Ara h3 and Ara h6) are displayed for A: 1 µg/ml (n=20); B: 10 µg/ml (n=16) and C: 100 µg/ml (n=10).

With the lowest concentration (1 µg/ml), SPT was mainly positive for Ara h6 (12/20) and in a minority positive for Ara h1 (3/20) and for Ara h2 (4/20). At a concentration of 10 µg/ml, SPT was again mainly positive for Ara h6 (13/15), but also for Ara h2 (9/15) and in a minority for Ara h1 (3/15) and for Ara h3 (1/12). At the highest concentration (100 µg/ml), SPT was positive for Ara h2 (9/9) and for Ara h6 (7/8), and in half of the subjects for Ara h1 (4/9) and for Ara h3 (5/9).

SPT were less often positive than the immunoblots, especially at the lower concentrations (1 and 10 µg/ml). At the highest concentration (100 µg/ml), the *in vivo* reactivity to major allergens detected by SPT was similar to the recognition of these allergens on immunoblot in 26 of the 35 performed SPT (74%). At this concentration, 3 children had a positive SPT for Ara h1, Ara h3 or Ara h6, whereas no recognition was observed on the immunoblot for these allergens.

Table I. Age and results of DBPCFC per subject, followed by the peanut-specific IgE and the IgE reactivity to major peanut allergens on blot at t=0 and t=20 months.

no	age	DBPCFC			Immunoblot t=0 months				
		severity†	ED‡	IgE*	Ara h1	Ara h2	Ara h3	Ara h6	Tot$
pi40	12	1	1000	3.3	-	+	-	-	1
pi29	4	1	1000	15	-	++	++	+	3
pi22	10	1	300	61	+	+++	+++	++	4
pi17	6	1	10	527	+++	++++	+++	++++	4
pi36	7	1	10	163	++	++++	-	+++	3
pi15	14	2	>3000	3.0	+	+	-	-	2
pi38	7	2	>3000	21	++	++	+	+++	4
pi9	6	2	3000	6.9	-	+	-	+	2
pi16	15	2	3000	36	-	+	-	+	2
pi20	5	2	3000	4	-	+	-	+	2
pi41	7	2	3000	35	-	++	-	-	1
pi25	4	2	1000	1.0	-	+	-	+	2
pi27	3	2	1000	7.5	+	+	+	+	4
pi21	12	2	300	21	-	+++	+	+++	3
pi39	4	2	300	2.5	-	+	-	+	2
pi11	5	2	100	250	+	++++	+	+++	4
pi37	12	2	100	3.3	-	+	-	-	1
pi24	4	3	1000	815	+++	++++	++++	++++	4
pi28	5	3	1000	99	+	+++	+	+++	4
pi19	6	3	1000	183	+	++++	++	+++	4

* Specific IgE in kU/l
† Severity: 1 = mild; 2 = moderate; 3 = severe
‡ ED = eliciting dose [mg peanut flour]
$ Tot=total number of allergens recognized by each individual

No clinical implications of major allergen recognition

It has been suggested that the severity of peanut allergy is related to the number of allergens recognized. [12,13] It appeared that the severity of the allergic reaction to peanut determined by DBPCFC was not correlated with the extent of allergen recognition, nor with the number of allergens recognized, neither by immunoblot nor by SPT. The ED was not correlated with these parameters either.

Discussion

Ara h2 and Ara h6 appear to be the most frequently and most strongly recognized major peanut allergens in peanut allergic children, as determined both by immunoblot and SPT. The recognition of all major allergens remained stable 20 months after DBPCFC with peanut.

no	DBPCFC		Immunoblot t=20 months					
	severity†	ED‡	IgE*	Ara h1	Ara h2	Ara h3	Ara h6	Tot$
pi40	1	1000	2.1	-	+	-	+	2
pi29	1	1000	24	-	+++	++	+	3
pi22	1	300	46	-	+++	+++	++	3
pi17	1	10	616	+++	++++	++++	++++	4
pi36	1	10	309	+++	++++	++	++++	4
pi15	2	>3000	7.8	+	+	-	-	2
pi38	2	>3000	44	+	++	+	+++	4
pi9	2	3000	5.0	-	+	-	+	2
pi16	2	3000	23	-	++	-	+	2
pi20	2	3000	3.8	-	+	-	+	2
pi41	2	3000	46	+	+	-	-	2
pi25	2	1000	1.0	-	+	-	+	2
pi27	2	1000	9.5	-	++	+	++	3
pi21	2	300	18	-	+++	+	+++	3
pi39	2	300	1.9	-	+	-	-	1
pi11	2	100	67	+	++++	+	+++	4
pi37	2	100	1.9	-	+	-	+	2
pi24	3	1000	606	+++	++++	++++	++++	4
pi28	3	1000	54	+	+++	+	+++	4
pi19	3	1000	163	+	++++	++	+++	4

* Specific IgE in kU/l
† Severity: 1 = mild; 2 = moderate; 3 = severe
‡ ED = eliciting dose [mg peanut flour]
$ Tot=total number of allergens recognized by each individual

To our opinion this is the first study that investigated the IgE reactivity to 4 major peanut allergens simultaneously in children with confirmed peanut allergy. Ara h1 and Ara h2 have long been regarded as the most potent peanut allergens, as they are recognized by the majority of peanut allergic subjects. [5-9] Comparative studies between both allergens acknowledged the importance of Ara h2 over Ara h1 [5;7;8].In the current study, Ara h6 seems to have a similar allergenic IgE binding potential as Ara h2. This is in line with recent studies in adults from our group. [11;12] In contrast to other studies, we used purified allergens in addition to CPE. [6;9] In this way, we were able to differentiate the IgE reactivity to these major allergens on the immunoblots, especially in the region of 14 – 22 kDa where several peanut allergens are located. IgE reactivity to Ara h2 (17 and 20 kDa) and Ara h6 (15 kDa) was present in almost all children with peanut allergy.

To illustrate IgE binding with *in vivo* reactivity to these peanut allergens, we also performed SPT with these purified allergens. This procedure turned out to be a safe and relatively easy technique

in children. SPT was mainly positive for Ara h2 and Ara h6, of which Ara h6 induced the strongest reactivity. Ara h6 can be seen as a truncated isoform of Ara h2, missing one IgE-binding epitope, and is therefore believed to be less potent. However, it has been observed before that Ara h6 shows a unique capacity of inducing SPT reactivity, which may be explained by a better exposure of IgE-binding epitopes in the 3D structure of our purified Ara h6 than predicted by amino-acid sequence.[12] Discrepancies between immunoblot and SPT were noted in a minority of the tests (26%). A possible explanation is the difference in the number of epitope binding between mast cells and immunoblot. Mast cells need at least 2 epitopes on the same allergen for cross-linking of IgE, whereas one epitope for IgE binding on the blot is sufficient. [22] On the other hand, (conformational) epitopes may be lost during the electrophoresis used for immunoblotting, rendering only the SPT positive. Overall, SPT confirmed the importance of Ara h2 and Ara h6 as was found by immunoblotting.

Both Ara h2 and Ara h6 are 2S albumins and show great homology; therefore IgE binding epitopes may be similar. [23] Cross-reactivity between Ara h2 and Ara h6 has been suggested before. [11] The immunodominant epitopes in Ara h2 and Ara h6 are very resistant to enzymatic digestion, which may enhance their allergenicity. [24;25] In general, IgE reactivity to 2S albumins is a risk factor for more serious reactions. [26] As has been shown in this study, these allergens in peanut show profound IgE binding and SPT reactivity and therefore the development of future therapeutic intervention strategies may be focussed predominantly on Ara h2 and Ara h6. Previous studies suggested that the number of allergens recognized, so-called ''promiscuity of IgE'' was a risk factor for more serious reactions. [12;13] No correlation between the severity of the reaction during DBPCFC and the number of purified allergens recognized was found in this study, neither by immunoblot, nor by SPT. The total performed SPT at 100 µg/ml in our study may be a limiting factor. We did see a correlation between the number of major allergens recognized and the level of specific IgE, which is in accordance with the study of Lewis et al. [13]

Despite the single exposure to peanut during DBPCFC, specific IgE levels remained stable. Moreover, the individual IgE binding pattern to Ara h1, Ara h2, Ara h3 and Ara h6 turned out to be highly comparable between before and 20 months after DBPCFC. During the observation period, 13 children had a less strict dietary management, making exposure to peanut rather likely. This is in accordance with the study of Van Odijk et al., who reported stable peanut specific IgE levels irrespective of exposure to peanut. [15] At least 6 children reported a reaction after ingestion of peanut containing products. Neither the dietary management nor the accidental reactions to peanut were related to any changes in IgE reactivity. In 6 children, we noticed minimal differences in IgE reactivity between the two time points. Because analyses of both time points were performed

simultaneously on a similar blot, differences were accounted to individual changes rather than changes in the procedure. An explanation could be that the recognition of major allergens on the blot was around the detection limit, resulting in no (-) or very low (+) IgE reactivity. Only one child developed marked IgE binding to Ara h3 after 20 months. As far as the history allowed, no different dietary management or accidental ingestions of peanut were reported in this child. Therefore, we conclude that IgE reactivity remained stable in all children after 20 months, despite DBPCFC.
This suggests that after an initial increase in specific IgE levels early in life, the levels and also the recognition of the specific major peanut allergens remain highly stable. For future research on the development of initial sensitization to peanut and possible interference by therapy, it may be interesting to investigate the sensitization to Ara h2 and Ara h6 in younger children.

Conclusion

Ara h2 and Ara h6 are the most frequently recognized major peanut allergens in children with peanut allergy, which is comparable to adults. The individual recognition pattern remained stable after challenge and subsequent elimination during 20 months.

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Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy

Abstract

Background: Better understanding of the relationship between antibody response to peanut and clinical sensitivity may lead to more accurate prognostication.

Objective: To investigate peanut-specific IgE and IgG4 epitope diversity in relation to challenge-defined clinical sensitivity to peanut in a group of peanut-sensitized children.

Methods: Clinical sensitivity was determined by double-blind placebo-controlled peanut challenges (DBPCFC) in 24 sensitized children. Six atopic controls were included. Specific IgE and IgG4 binding to 419 overlapping 15 amino-acid peptides representing the sequence of recombinant Ara h1, Ara h2 and Ara h3, was analyzed by peptide micro-array immunoassay (MIA).

Results: Peanut-sensitized patient sera bound significantly more IgE and IgG4 epitopes than control sera. This patient group reacted to the same major Ara h1, Ara h2 and Ara h3 epitopes as reported previously. There was a positive correlation between epitope diversity (i.e. number of epitopes recognized) and clinical sensitivity ($r=0.6$) such that patients with the greatest IgE epitope diversity were significantly more sensitive than those with the lowest IgE diversity ($p=0.021$). However, no specific epitopes were associated with severe reactions to peanut. IgG4 binding was observed to largely similar epitopes, but was less pronounced than IgE binding, and did not relate to the clinical sensitivity to peanut. IgE and IgG4 epitope recognition patterns were largely stable over a period of 20 months.

Conclusion: Clinical sensitivity, as determined by DBPCFC, is positively related to a more polyclonal IgE response, which remains stable over time.

Clinical implications: Children with more severe allergic reactions to peanut can be characterized by greater diversity of IgE epitopes.

Introduction

Peanut allergy is among the most prevalent and severe food allergies in westernized countries. [1;2] Previous studies suggest that clinical sensitivity to allergens including peanut, may be related to allergen-specific IgE epitope pattern, diversity and avidity, all of which likely play a role in IgE function on effector cells such as mast cells and basophils. [3-7]

Numerous peanut allergens (designated Ara h 1-8) [8] have been characterized and the sequential IgE binding epitopes of peanut allergens have been previously defined for several of them. [9-13] Several studies have shown in both peanut-allergic patients and sensitized animals that the majority of the IgE response is directed to Ara h 1, Ara h 2, Ara h 3, and Ara h 6. [3; 14; 15]

The function of allergen-specific IgE may also be modulated by the presence of other specific antibody classes. Elevated specific IgG4, in particular, has been associated with suppression of IgE-dependent immediate hypersensitivity reactions, e.g. in the context of venom immunotherapy, natural high dose cat exposure and helminth infection. [16-18] Allergen-specific IgG subclasses have also been shown to suppress IgE-facilitated antigen presentation suggesting that they may modulate the progression of adaptive immunity with ongoing allergen exposure. [19]

We showed previously that IgE diversity to continuous epitopes of Ara h 1-3 correlated with reaction severity as well as *in vitro* effector cell degranulation. [4] However, clinical reactions were not defined by double-blind placebo-controlled peanut challenge (DBPCFC) and the study was limited by its retrospective design. Lewis et al. also demonstrated a relationship between IgE diversity, defined by immunoblot, and clinical sensitivity. [3]

The aim of the present study was to prospectively determine the relationship between peanut allergen epitope-specific IgE and IgG4 diversity and clinical sensitivity defined by DBPCFC.

Methods

Study population

Twenty-four children (8 female and 16 male; age 3-15 years; mean 7.2 years) were recruited from the Department of Pediatric Dermatology/Allergology at the University Medical Centre Utrecht. These children participated in a previous study to determine eliciting doses (ED) for peanut and were a representative sample from the population of peanut-sensitized children in our outpatient clinic with regard to specific IgE levels and severity of previous allergic reactions to peanut. [20] Inclusion criteria consisted of elevated peanut-specific IgE. Six atopic, but not peanut-sensitized children

were included as controls. All parents gave written informed consent before enrollment in the study. The study was reviewed and approved by the Central Committee of Human based Research in The Netherlands (CCMO, The Hague, The Netherlands).

DBPCFC

DBPCFC was performed as described previously. [20] In short, increasing amounts of defatted peanut flour were given with time-intervals of 15-30 minutes: 0.01, 0.1, 0.3, 0.5, 1, 10, 100, 300, 1000 and 3000 mg. Four placebos were interspersed randomly. An open challenge of 10 peanuts was the last step in the procedure. Although all challenges were continued until there were objective symptoms, the first dose to provoke symptoms, either subjective or objective, was used for determination of the ED. The eliciting dose was scored inversely, with the lowest ED scored as 6, and the highest ED as 1 (Table I).

The severity of allergic reactions was scored according to Mueller in mild (grade 0 and 1), moderate (grade 2) and severe (grade 3 and 4). [21] Mild reactions were symptoms of the oral cavity, the skin and/or mucous membranes (urticaria, angio-edema, hoarseness, rhinitis, conjunctivitis), moderate reactions included gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain), and severe reactions consisted of respiratory symptoms (bronchoconstriction, stridor) and/or cardiovascular symptoms (Table I).

Peanut-specific IgE levels

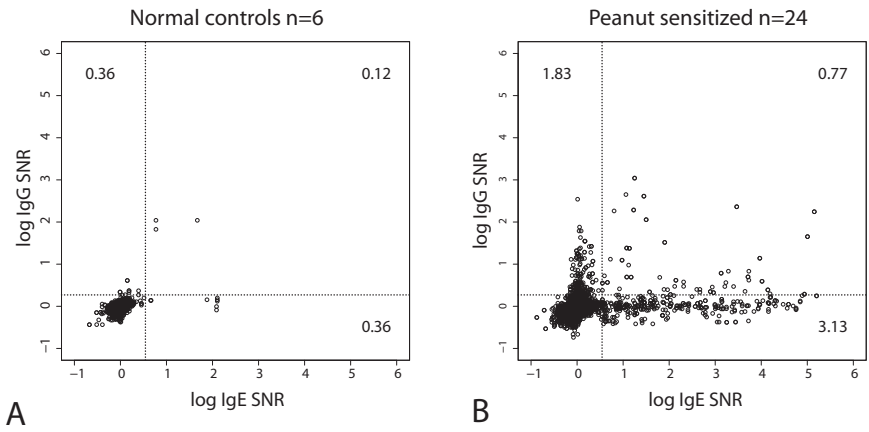
Serum samples were collected for each patient before and 20 months after DBPCFC. Peanut-specific IgE was determined in all samples by CAP-system FEIA according to manufacturer's instructions (Phadia, Uppsala, Sweden). Specific IgE > 0.35 kU/l was considered positive.

Peptides, reagents, and array production

We determined IgE and IgG4 binding to sequential epitopes of Ara h 1, Ara h 2 and Ara h 3 using a peptide micro array-based immunoassay. [4; 13] A library of peptides, consisting of 15 amino acids length with an offset of 3, corresponding to the primary sequences of Ara h 1, Ara h 2 and Ara h 3 were commercially synthesized (JPT Technologies GmBH, Berlin, Germany). Peptides included a four residue SGSG linker sequence and a reactive group for site-specific binding.

Stock solution of peptides were dissolved in dimethyl sulfoxide, to 0.2 mg/ml. Working dilutions were then made in Protein Printing Buffer (PPB, ArrayIt, Sunnyvale, CA, USA) to a final concentration of 150 μM and stored in 384-well polypropylene plates (Matrix Technologies, Hudson, NH, USA).

Figure 7.1



IgE and IgG4 peptide binding to peanut allergen derived peptides is significantly greater in peanut-sensitized patient versus control sera. IgE signal-to-noise ratio (SNR) versus IgG4 SNR. Dashed lines indicate cutoffs (see Methods). Numbers indicate percent above cutoff. A) control sera (n=6); B) peanut-sensitized sera at baseline (n=24).

Peptides were printed in triplicate to epoxy-derivatized glass slides (SuperEpoxy Substrate, ArrayIt, Sunnyvale, CA, USA) using NanoPrint™ Microarrayer 60 (TeleChem International, Inc., Sunnyvale, CA, USA). Slides were stored at 4° C until use. Additional array elements including purified Ara h 1 and whole peanut extract were used as positive controls. PPB alone was used as negative control and for background normalization. Fluorochrome-labeled bovine serum albumin elements were used for the purpose of grid alignment (positional control). All array elements were printed in duplicate (two sets of duplicates) to improve precision, and to determine intra-assay variation.

Immunolabeling

An area around the printed arrays was delimited with a hydrophobic PAP pen (DakoCytomation Pen, Dako, Denmark). The slides were rinsed with phosphate-buffered saline containing 0.5% tween 20 (PBS-T) and non-specific binding sites were blocked with 1% human serum albumin in PBS-T for 1 hour (PBS-T/HSA). After removing the PBS-T/HSA from the slide surface, 80 µl of patient sera diluted 1:6 in PBS-T/HSA was incubated for 1 hour on a rotator. Polyclonal goat anti-human IgE diluted 1: 5000 (gift from Phadia, Uppsala, Sweden) combined with monoclonal mouse anti-human IgG4 diluted 1:10000 (Pharmingen, clone G174, San Jose, CA, USA), which have been covalently tagged with Alexa 546 and Alexa 647 (Molecular Probes – Invitrogen, Carlsbad, CA,

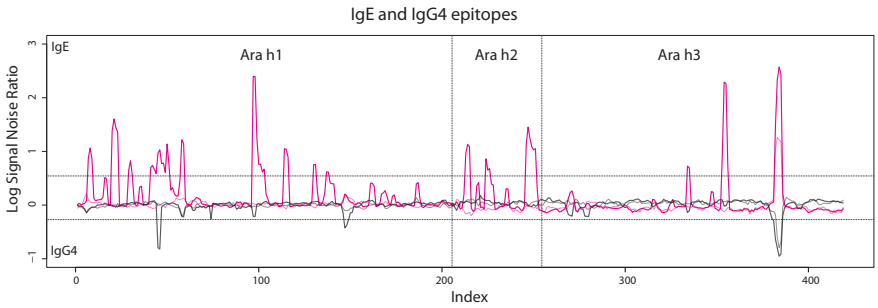
USA) respectively, were then incubated for 1 hour. All incubations were performed in a humidity chamber (Binding Site, Birmingham, UK), at room temperature in the dark. Slides were washed with PBS-T, centrifuged dry and scanned using a ScanArray®Gx (PerkinElmer,Waltham, MA, USA). Images were saved as TIF files. Fluorescence signal was digitized with the program ScanArray Express (Perkin Elmer, Waltham, MA, USA).

Analysis

Data were exported from ScanArray as comma-delimited text files which were related to array element ID and analyzed using R analysis. [22] The complete data set and all analyses and figures in R script are available through the repository files. The read out used for both IgE and IgG4 binding was the median fluorescence signal-to-noise ratio (SNR) of the four replicates for each array element. A complete R script for all analysis and production of figures from raw data along with raw data files are available in the online repository. Briefly, median SNR values were $\log^2$ transformed and median-centered for inter-slide comparison. Array features with intraslide CV > 5% were excluded from further analysis (<1% of total). Based on our previous data [13] and the published literature on B cell epitope length, we defined positive binding as necessarily involving at least two overlapping peptides (i.e. maximum length 12 residues) and therefore subsequent analysis was carried out on data for each element adjusted to the median of itself and the two flanking peptides (see Repository Figure 1 for display of data without this 'near neighbor normalization'). Repository Figure 2 shows histograms for IgE and IgG4 data derived from control sera. For calculation of percent positive for each peptide, cutoff values for IgE and IgG4 were determined from these control data at p=0.995 by the formula $p(k) = (k - 1/3) / (n + 1/3)$. The resulting probability estimates are approximately median-unbiased regardless of the distribution of the data.

We defined the clinical sensitivity as the product of the Mueller symptom score and the eliciting dose score (Table 1), and this was treated as a ranked variable. For correlation of either clinical sensitivity, symptom score or eliciting dose score with epitope number, Spearman's ranked correlation test was used. For comparison of clinical sensitivity score between the highest and lowest epitope quartile groups, the Wilcoxon test was used. Chi-squared test was used to assess the significance of agreement of IgE and IgG4 binding patterns over time.

Figure 7.2



Multiple sequential epitope rich regions within the peanut allergens, Ara h 1-3. A) Representation of mean log SNR for binding of patient IgE (orange line) and IgG4 (dark grey line) versus control (grey lines). Dashed lines indicate cutoff values (see Methods).

Results

IgE and IgG4 reactivity to peptides in healthy controls and study subjects

IgE and IgG4 binding to peptides from Ara h 1, Ara h 2 and Ara h 3 was detected simultaneously using 2 distinct fluorescence channels. For the non-allergic control subjects, IgE and IgG4 fluorescence was low and by definition, signal above the arbitrary cutoff was low (<0.5% for both, Figure 1A, see Methods for cutoff determination). Using the same cutoffs for the patient group, 3.1% for IgE (312 positive peptides of a total number of 9967 peptides studied in the patient group) and 1.8% for IgG4 (182 of 9967 peptides) had signal above cutoff levels for each channel alone and an additional 0.8% (77/9967) were positive for both IgE and IgG4 (Figure 1B).

The number of positive IgE epitopes per individual ranged from 0 to 80 (median 9) and correlated with the level of peanut-specific IgE (r=0.579, Repository Figure 3). Overall, 22 of 24 (92%) patients recognized at least one epitope from the three allergens. IgE and IgG4 binding was clustered to epitope rich areas (Figure 2A). There were 17 distinct IgE signal maxima in Ara h 1, five in Ara h 2, and five in Ara h 3. One area in Ara h 3 not previously investigated by microarray assay, [4] was positive across a range of peptides with only three overlapping residues, IYR, for IgE and IgG4 in both the study group and controls, and is a suspected artifact of unknown significance. The IYR sequence was not present in other peptides. Excluding this sequence, the most dominant epitopes (Ara h 1 – **PSHQQPRKI, FYFPSRRFS**; Ara h 2 – **RRCQSQ;** Ara h 3 – **EDEYEYDEE**) were recognized by 30-40% of patients, consistent with previous results. [4]

Table I. Age, sex, specific IgE to peanut and results of DBPCFC per subject, followed by the calculation of the combination score as symptom score * ED score.

No	Age	Sex	IgE*	Symptoms†	Score	ED‡	Score	Combination
Pi5	3	male	4	-	0	-	0	0*0=0
Pi9	6	male	7	Rc, vom	2	3000	2	2*2=4
Pi11	5	female	250	Rc, hoa	2	100	5	2*5=10
Pi12	10	female	50	-	0	-	0	0*0=0
Pi14	6	male	0.4	Oas	1	3000	2	1*2=2
Pi15	14	male	2.7	Oas, rc, ap	2	>3000	1	2*1=2
Pi16	15	female	35	Oas, rc, vom	2	3000	2	2*2=4
Pi17	6	male	527	Oas, vom	2	10	6	2*6=12
Pi19	6	female	183	Rc, vom, bro	3	1000	3	3*3=9
Pi20	5	female	4	Oas, rc, ap, dia, hoa	2	3000	2	2*2=4
Pi21	12	male	20	Oas, rc, ap, vom	2	300	4	2*4=8
Pi22	10	female	61	Oas, ap, vom, dia	2	300	4	2*4=8
Pi24	4	male	815	Gu, vom, str	3	1000	3	3*3=9
Pi25	4	male	1	Oas, fs, ap	2	1000	3	2*3=6
Pi26	5	female	48	Gu, rc, vom	2	3000	2	2*2=4
Pi27	3	male	8	Oas, fs, rc, ap, hoa	2	1000	3	2*3=6
Pi28	5	male	99	Oas, gu, rc, bro, tach	3	1000	3	3*3=9
Pi29	4	female	15	Oas, ap, vom	2	1000	3	2*3=6
Pi36	7	male	163	Oas, ap	2	10	6	2*6=12
Pi37	12	male	3	Oas, fs, urt, rc, ap	2	100	5	2*5=10
Pi38	7	male	93	Gu, rc, ap, vom, dia	2	>3000	1	2*1=2
Pi39	4	male	2	Oas, vom, rc	2	300	4	2*4=8
Pi40	12	male	3	Nau, vom	2	1000	3	2*3=6
Pi41	7	male	35	Oas, cu, fs	1	3000	2	1*2=2

* Specific IgE in kU/l
† symptoms: oas=oral allergy syndrome, cu=contact urticaria, urt=urticaria, gu=generalized urticaria, fs=facial swelling, c=conjunctivitis, rc=rhinoconjunctivitis, hoa=hoarseness, ap=abdominal pain, vom=vomiting, dia=diarrhea, str=stridor, bro=bronchoconstriction, tach=tachycardia
‡ ED = eliciting dose [mg peanut flour]

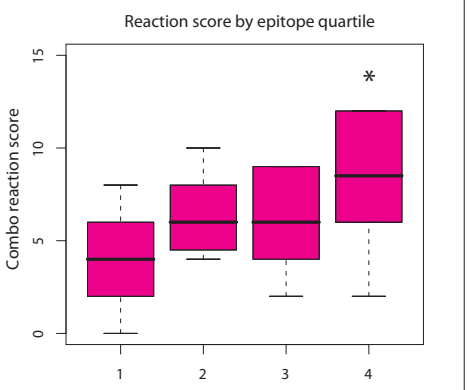
Clinical evaluation of peanut allergy

In order to relate the IgE and IgG4 binding epitopes to the clinical reactivity to peanut, the severity of the symptoms and the eliciting dose (ED) were determined by DBPCFC as previously described.[20] Challenge outcomes are displayed together with demographic data and peanut-specific IgE levels in Table I. Twenty-two children (92%) developed an allergic reaction to peanut during DBPCFC, ranging from isolated oral pruritis to multi-systemic reactions with severe bronchospasm, generalized urticaria and tachycardia.

The individual ED ranged from 10 mg to greater than 3 g. In order to take both parameters into

account, we utilized a scoring for the clinical sensitivity according to a combination of severity of symptoms (mild, moderate, severe) and eliciting dose (step 1-6, corresponding to reactions from > 3000 mg to reactions from 10 mg). The symptom score was multiplied by the eliciting dose score to give a 'combination score' conceptually similar to Lewis et al. (Table I). [3]

Figure 7.3



Greater IgE binding corresponds to greater clinical sensitivity. Subjects were binned into quartiles according to the number of IgE epitopes bound. The group with the greatest IgE binding (4) had significantly higher combination scores in comparison to those with the least IgE binding (1) (*; p=0.021).

IgE reactivity to peptides in relation to the clinical sensitivity to peanut

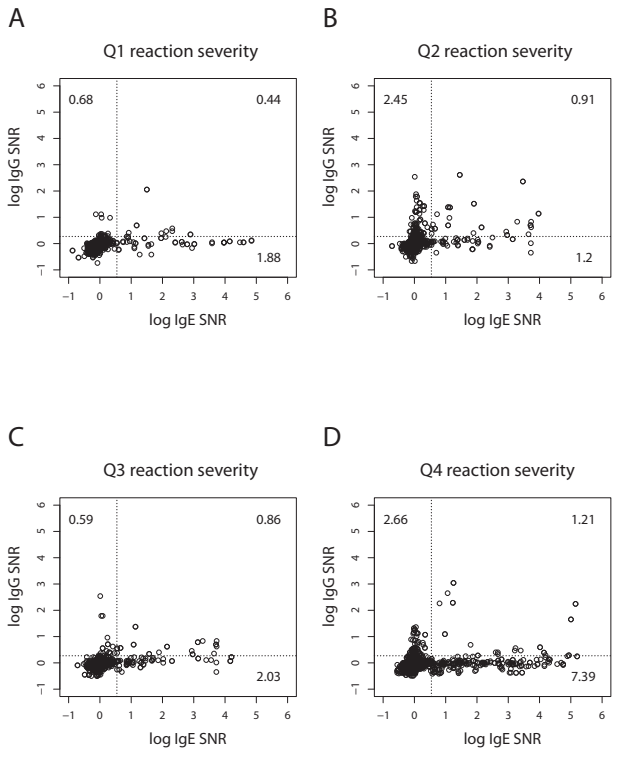
Specific IgE binding regions for children with either low or high combination scores were not found. Individual epitope binding patterns were variable and unique (Figure 2B). A positive relationship between the clinical sensitivity to peanut, defined by the combination score, and number of positive IgE epitopes was found (Spearman r = 0.58). When subjects were binned into quartiles by the number of recognized epitopes, a trend was observed for higher clinical sensitivity with greater IgE epitope diversity, which reached statistical significance for the comparison between the highest and lowest quartiles (Figure 3A, median combination score [range] = 8.5 [2-12] vs. 4 [0-8] for group 4 and 1 respectively; Wilcoxon p=0.0214). IgE and IgG4 reactivity were also compared after grouping by combination score (Figure 4). The quartile of patients with the highest clinical sensitivity had positive IgE binding to ~7.4% of all peptides compared with 1.9, 1.2 and 2.0% for the first, second and third quartile groups respectively. Two children appeared not clinically allergic upon challenge, including one with a level of specific IgE previously determined to be highly suggestive of clinical reactivity (Pi12, 50 kU/L, Table 1). [23] Both of these individuals had very little epitope binding by microarray assay. In the case of Pi12 there were no positive epitopes, for Pi5, only Ara h 3 IYR was positive. Analysis excluding these two non-allergic individuals did not change the observed relationship between IgE

Epitope diversity and higher combination scores.

One patient, Pi38, with high IgE levels and a very polyclonal epitope pattern (41 positive epitopes), was graded as a mild reactor by the combination score as his symptoms were limited to skin and GI tract (Mueller grade 2) at a high eliciting dose (>3 g). However, this child developed generalized urticaria after experiencing a complete loss of bowel control and was treated with epinephrine by clinical staff for his sick appearance.

The relationship between IgE binding and combination score was stronger than that of IgE binding to either symptom score alone (r=0.49) or eliciting dose alone (r=0.32). IgE epitope number correlated more strongly with clinical sensitivity than specific IgE (r=0.58 vs. r=0.52).

Figure 7.4



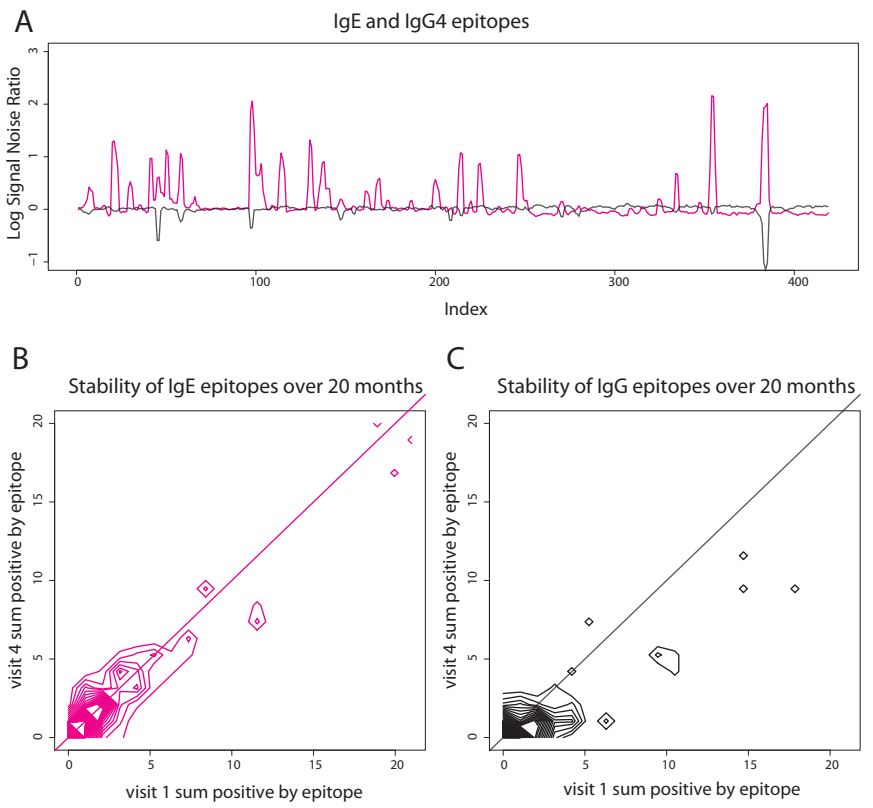
The most clinically sensitive patients recognize the most IgE epitopes. IgE versus IgG4 SNR is shown in separate plots by quartile of clinical sensitivity (A-D for least to most sensitive; see Methods). Dashed lines represent cutoff. Numbers in each quadrant represent percent above cutoff.

IgG4 reactivity in relation to clinical sensitivity

The number of IgG4 epitopes varied from 0 to 64 per subject (median 6). IgG4 epitopes were mainly located on Ara h 1 and Ara h 3, with epitope densities of 2.2% (percentage of peptides with SNR above cutoff level) and 3.5% respectively. In contrast, Ara h 2 had a density of IgG4 epitopes of 1.0%. No relationship was observed between IgG4 diversity and clinical sensitivity.

IgG4 binding coincided with IgE binding in 29.6% of the IgG4 positive peptides (Figure 1). Consistent with previous analysis of Ara h 2, [13] this was more than expected on the basis of the frequency of IgE and IgG4 alone (0.77% double positive observed versus 0.1% expected; χ^2 test; $p<0.001$).

Figure 7.5



IgE and IgG4 binding patterns are largely stable over 20 months. Correspondence between baseline and 20-month follow up is shown as a topographical representation of the number of patients that are positive for each peptide before (x-axis) and 20 months after DBPCFC (y-axis); n=22. A) IgE; B) IgG4. Diagonal represents identity between t=0 and t=20 months.

IgE and IgG4 reactivity after 20 months

Previously, we demonstrated that the IgE binding to the major peanut allergens by Western immunoblot was not changed at 20 months after DBPCFC in these subjects. [24] To determine the stability of the epitope diversity over time, MIAs were repeated with serum samples obtained 20 months after the challenge. At that time point, from 2 subjects no serum samples were available. For the remaining 22 children, in general, epitope diversity was highly similar (χ^2 test; $p<0.001$ for frequency of agreement between baseline and 20 months for both IgE and IgG4; Figure 5). IgE binding patterns were more stable over time than IgG4. We did not observe a substantial change in IgE epitope diversity in any of the subjects 20 months following challenge.

Discussion

The goal of the present study was to investigate in peanut-allergic children the IgE and IgG4 peanut allergen epitope recognition in relation to clinical reactivity as determined by DBPCFC. We found that children with more severe reactions recognized more diverse IgE epitopes suggesting a more polyclonal response, whereas IgG4 epitope recognition was less abundant and did not relate to clinical sensitivity to peanut. Binding was clustered to previously described epitope-rich areas. However, as we observed before using this method, each individual displayed a unique 'fingerprint' of IgE and IgG4 binding and these individual binding patterns remained largely stable 20 months later.

The peptide micro-array immunoassay (MIA) used in this study has been documented before as a reliable tool to describe sequential IgE epitopes on peanut allergens. [4;13] The reproducibility of this method was demonstrated here by the finding that IgE binding epitopes on Ara h 1, Ara h 2 and Ara h 3 were similar to the epitopes described in other peanut-allergic populations using different methods, [9;11;25] and the reproducibility of epitope recognition in these patients over time.

The majority of IgE epitopes were located on Ara h 1 and Ara h 2. The dominant IgE binding to Ara h 2 derived peptides was in line with previous findings using Western immunoblots, showing that Ara h 2 is an important peanut allergen. [3;24] Ara h 2, and its homologue Ara h 6, are 2S albumin family members with compact conformations that remain stable during heating and digestion, which may contribute to their allergenicity. [26;27;28] In the present population, multiple IgE binding epitope regions on Ara h 1 were defined as well. This is consistent with previous mapping data but contrasts somewhat with results obtained by SPT and Western immunoblot in the current peanut-allergic population. [24] Differences between the sequential structure of Ara h 1 in the current study

compared to tertiary structure of purified Ara h 1 may be an explanation. Another difference between purified Ara h 1 and the peptides used in the current study, that were derived from a recombinant Ara h 1, is that a fragment of 86 aa on the N-terminus is cleaved off during synthesis. Several epitopes are located on this fragment. [12] Excluding these epitopes demonstrated that the results from MIA and the western immunoblot were in agreement (not shown). It has been reported that proteolytic fragments after enzymatic digestion of Ara h 1 retained IgE binding epitopes. [29] This may suggest the relevance of sensitization to both Ara h 1 and Ara h 2 in peanut allergic subjects, which has been implied before for peanut allergic children. [15;30]

A significant strength of this study over previous investigation of IgE epitopes in relation to clinical sensitivity is the fact that all subjects were characterized prospectively by DBPCFC. In order to define clinical sensitivity, we combined the severity of symptoms and the ED. As Hourihane and colleagues have suggested, the ED is a clinically relevant parameter in combination with the severity of the allergic symptoms. [3;31] Interestingly, IgE epitope diversity correlated better with the combination score than with either symptom score or ED score alone.

The diversity of IgE binding was unique for each subject, and IgE epitopes were recognized by a maximum of 30-40% of subjects. Therefore, no specific immunodominant regions could be related to more severe reactions. Previous studies have also suggested that the number of allergens recognized by IgE rather than the recognition of particular allergens was a risk factor for more serious reactions. [3;32] Our findings underline that the polyclonality of the antibody response is related to the clinical sensitivity to peanut. Presentation of clustered epitopes may facilitate IgE cross-linking and basophil degranulation. [7] In support of this, we previously showed that serum containing IgE to a larger number of IgE epitopes on peanut lowered the threshold for basophil and mast cell degranulation. [4] The diversification of epitope recognition through a process of epitope spreading is a regulated T cell dependent process that proceeds in concert with affinity maturation. [33;34]

In contrast to the relatively high frequency of IgE epitopes recognized in these patients, IgG4 binding was low. We hypothesized that IgG4 epitope number might inversely correlate with clinical sensitivity, as IgG4 has been shown in other contexts to inhibit the activity of IgE by competing for binding sites. [19] However, no association between IgG4 binding and clinical sensitivity was demonstrated. Somewhat surprising to us was the finding that IgG4 reactivity even in the two peanut-sensitized subjects without peanut allergy was nearly absent. These low levels of IgG4 may be a reflection of very low exposure to peanut during elimination diets prior to DBPCFC. as in the contexts where IgG4 has been correlated with protection, such as in cat allergy, it is

associated with chronic antigen exposure. [17] The relevance of IgG4 in peanut allergy needs to be further investigated and compared to other food allergens such as cow's milk, for which we did observe high levels of IgG4 reactivity with Western immunoblot [35] and with MIA (unpublished observations).

The peanut-specific IgE and IgG4 binding pattern remained largely intact 20 months later. In this period, exposure to peanut occurred during DBPCFC, and possibly exposure to trace amounts in the subsequent elimination period. Five children reported inadvertent ingestion of peanut in that period, without significant changes in the IgE or IgG4 profile (data not shown). Exposure to allergens has been suggested to mature the B cell response, with expanded serological and clinical reactivity. [36] For some clinicians and patients this potential maturation might even be a reason to avoid food challenges. The observation in this study indicates that the diversity of IgE and IgG4 binding to peanut was present before DBPCFC and did not develop into an enhanced polyclonal response after a 20-month period.

In conclusion, this study shows that the B cell response in children with more severe allergic reactions to peanut was characterized by a more diverse peanut-specific IgE response, directed mainly to Ara h 1 and Ara h 2. IgG4 reactivity was less dominant and did not relate to the severity of the clinical reactivity to peanut. Both IgE and IgG4 binding remained stable over a period of 20 months.

Acknowledgements

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Manuscript in preparation

T cell responses to major peanut allergens
in children with and without peanut allergy

8

Abstract

Background: T cell responses involved in peanut allergy are poorly understood.

Objective: To investigate T cell responses towards major peanut allergens and to a selection of potential T cell epitopes in peanut-allergic subjects compared to non-allergic controls.

Methods: Nineteen peanut-allergic children (PA), 7 non-allergic peanut-sensitized children (PS) and 11 non-atopic adults (NA) were included. PBMC were stimulated with crude peanut extract (CPE). Short-term T cell lines were generated and subsequently stimulated with CPE and purified Ara h1, Ara h2, Ara h3 and Ara h6. Proliferation and production of IL-13, IFN-γ, IL-10, and TNF-α were analyzed. A selection of potential T cell epitopes using computer algorithms was used in a PBMC stimulation assay in 11 PA and 16 healthy control subjects.

Results: Proliferation to CPE and major allergens was enhanced in PA subjects. The primary response to CPE was comparable with PS subjects, with increased production of IL-13 and IFN-γ compared to NA. Production of IL-10 was not observed. In short-term T cell lines, the response to CPE was stronger in PA than in PS and NA subjects. Only peanut-allergic children had a response to major peanut allergens, characterized by IL-13 production. The response was highest after Ara h3 stimulation, and lowest after Ara h2 stimulation. Several peptides derived from peanut allergens were able to induce a T cell response, especially in control subjects.

Conclusion: T cell responses to CPE in PA and PS children were characterized by Th1 and Th2 cytokines. Only PA children showed enhanced Th2 responses to Ara h1, Ara h3 and Ara h6. A selection of peanut peptides induced T cell responses.

Introduction

Peanut allergy is a serious disease which affects approximately 1% of the westernized population. Peanut-allergic children display a maladaptive immunological response to peanut, which is characterized by the production of peanut-specific IgE. However, specific IgE to peanut is not always clinically relevant. [1] According to recent figures from the UK, the prevalence of subjects sensitized to peanut (2.8%) exceeds the prevalence of peanut allergic subjects (1.8%). [2] Understanding of the underlying immunological mechanisms that determine allergy or tolerance to peanut remains limited. A previous report showed that T cells from non-allergic children and children that have outgrown peanut allergy produce predominantly Th1 cytokines, whereas peanut-allergic children display a Th2 profile, which is associated with atopy and the production of IgE. [3] Allergic children had overall higher proliferative responses than non-allergic subjects, suggesting an intrinsic excessive reactivity of the allergic subject's T cells. [4] However, a recent study demonstrated that the T cell response to peanut was not different for peanut-allergic and peanut-tolerant sensitized adults, both showing increased Th2 responses. [5]

Extensive knowledge about the serological IgE responses to the major peanut allergens and epitopes was gathered in the past few years. Previous results from our group showed that the B cell response from peanut allergic subjects was predominantly directed to Ara h2 and Ara h6. [6-8] However, information about the T cell response to the major allergens in peanut, rather than to crude peanut extract, is lacking. A step further is the analysis of T cell responses to peanut peptides in order to identify immunodominant epitopes that could possibly be used to induce tolerance in peanut-allergic subjects. [9] Because investigating T cell responses to all possible peptides is not feasible, a selection of peptides should be made in advance. In the present study, a computer algorithm predicting pan-HLA-DR-binding peptides on a given protein sequence was used to identify potential MHC-class II-restricted T cell epitopes. [10;11]

This study was primarily set up to investigate the T cell responses to crude peanut and to purified Ara h1, Ara h2, Ara h3 and Ara h6 in allergic children and to compare the outcome with non-allergic peanut-sensitized and non-atopic subjects. After selection of potential T cell epitopes by a computer algorithm, the response to these peanut peptides was also examined in allergic and non-allergic subjects.

Methods

Study populations

Three groups were recruited for the evaluation of T cell responses to peanut and major peanut allergens Ara h1, Ara h2, Ara h3 and Ara h6. The first group (5 female and 14 male; aged 3.4-15.0 years) comprised 19 peanut-sensitized children with peanut allergy (PA). Peanut allergy was previously confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). All children with peanut allergy had immediate symptoms during DBPCFC, and subsequently eliminated peanut from their diet. [12] The second group (4 male and 3 female; aged 4.4 – 14.0 years) consisted of 7 non-allergic, but peanut-sensitized children (PS). Four of these 7 children were recruited after negative DBPCFC and 3 currently peanut-consuming children were recruited from the outpatient clinic. At inclusion, sensitization to peanut was confirmed in all children using CAP-system FEIA. The PA and PS children were comparable with regard to age (mean 7.2 versus 9.9 years; p=0.483) and specific IgE levels (median 22.5 versus 14.1 kU/L; p=0.366). The third group (4 male and 7 female; aged 22-60 years) comprised 11 non-atopic adults (NA). The NA subjects had no elevated total IgE, no peanut-specific IgE and no clinical or family history of allergy or atopy. After informed consent was obtained, a heparinized venous blood sample was taken. PBMC were isolated by density gradient centrifugation with Ficoll (Amersham, Uppsala, Sweden) and cryopreserved until use.

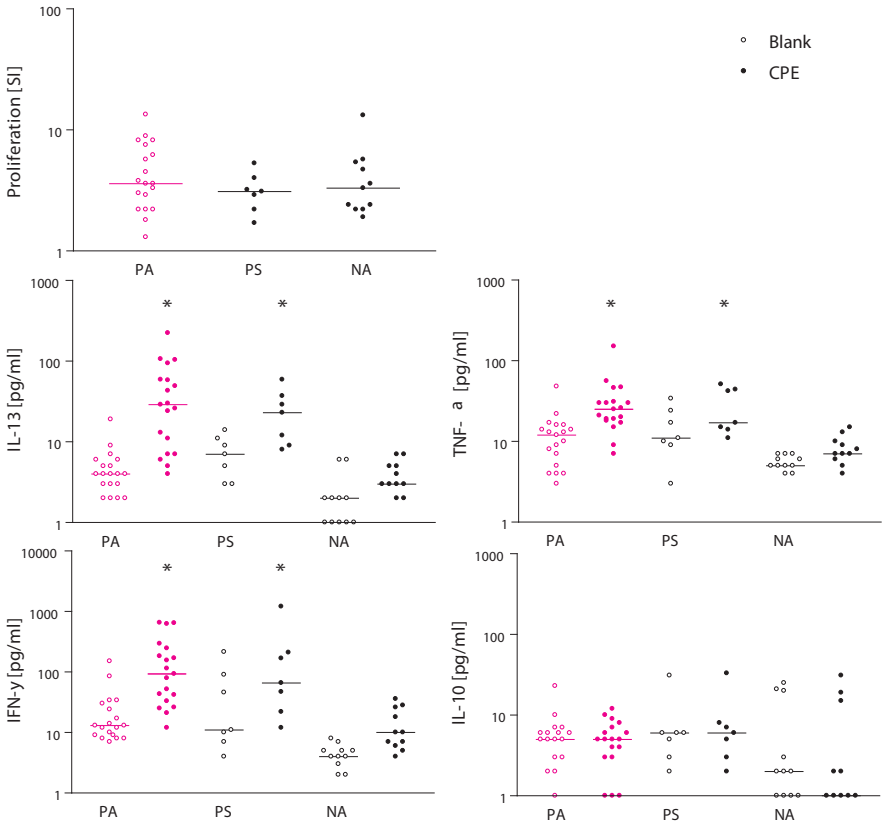
For the comparison of the T cell response to peanut peptides, PBMC were isolated from 4 PA children (3 male and 1 female; aged 6-12 years), 7 PA adults (2 male and 5 female; aged 19-42 years) and 16 adult healthy controls (HC; anonymous). The freshly isolated PBMC were directly used in the stimulation assay.

The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht.

Purified peanut allergens

Crude peanut extract (CPE) and purified allergens Ara h1, Ara h2, Ara h3 and Ara h6, were a kind gift from TNO Quality of Life, Zeist, the Netherlands. Previously developed purification protocols were used for the preparation of Ara h1, [6] Ara h2, [6] Ara h3, [13] and Ara h6, [7] with purity of >95% as judged by SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining. Individual peanut allergens were sterilized and stored as described before. [6] The peanut allergens were free of LPS contamination.

Figure 8.1



Proliferation (A) is shown as the stimulation index (SI) and IL-13 (B), IFN-γ (C) and TNF-α (D) production with (○) and without (●) stimulation with CPE in short-term peanut-specific T cell lines displayed for PA (orange), PS and NA subjects. Differences between blank and CPE were based on Wilcoxon matched pairs test: * $p<0.05$.

Selection of peanut peptides

Information on protein sequences of various recombinant isoforms of Ara h1, Ara h2, Ara h3 and Ara h6 were derived from the SwissProt protein knowledge database (accession codes P43238 for Ara h1, Q8GV20 for Ara h2, Q8LKN1 and Q5I6T2 for Ara h3, and Q9SQG5 for Ara h6). A computer algorithm based on HLA-DR1, HLA-DR4 and HLA-DR7 binding was used to predict pan-DR-binding epitopes (as described by Dr. A. Sette, LIAI, La Jolla, CA, USA) in these different peanut proteins. [11] The selection was narrowed down to 26 peptides spread over the peanut allergens Ara h1 (n=10), Ara h2 (n=4), Ara h3 (n=8) and Ara h6 (n=4) based on the predicted binding scores

(Table I). Crude preparations of these peptides (aimed at >70% purity) were synthesized as 9-mers containing the potential epitopes with three flanking residues at each side, by resin-based FMOC chemistry (Pepscan Systems BV, Lelystad, The Netherlands). Peptides were checked by HP/MS, and HPLC purified.

Lymphocyte stimulation tests

PBMCs were thawed and cultured in triplicate (2 x 10^5 cells/well) for 6 days in 96-well U-bottom culture plates (Greiner, Frickenhausen, Germany) in IMDM/5% human serum (Cambrex, Verviers, Belgium), supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), and glutamine (1 mM) (Gibco, New York, NY, USA). Culturing was performed in the absence or presence of CPE (50 µg/ml). In the peanut peptide assay, fresh PBMC were stimulated in triplicate with the selected peptides at a concentration of 20 µg/ml. After 6 days, supernatants were harvested and stored at -20°C for cytokine measurements. Tritiated thymidine ([^{3}H]-TdR, 1 µCi/well, Amersham, Aylesbury, UK) was added to the cultures, and the cells were harvested after 18 hours. Incorporation of [^{3}H]-TdR was measured using a 1205 z-plate counter (Wallac, Turku, Finland), and expressed in counts per minute (cpm). The stimulation index (SI) was defined as cpm of stimulated cells divided by cpm of cells cultured with medium alone. The SI was considered positive for CPE when the SI > 2.0, and for peptides when the SI > 1.8.

Generation of short-term peanut-specific T cell lines

For short-term T cell lines, 0.5 x 10^6 thawed PBMC/well were cultured in 48-well flat-bottom plates, in the presence of CPE (50 µg/ml). At day 7, IL-2 was added (10 IU/ml). At day 11, T cell blasts were isolated by Ficoll density centrifugation, and were expanded by adding irradiated allogeneic PBMC (2 donors, 0.5 x 10^6 PBMC/well) and 1 x 10^5 EBV-transformed B cells, in the presence of PHA (0.5 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) and IL-2 (10 IU/ml; kind gift from Novartis Research Institute, Vienna, Austria). At day 21, T cells (2 x 10^4/well) were stimulated with irradiated autologous PBMC (1 x 10^5/well) in the presence of CPE or purified Ara h1, Ara h2, Ara h3, Ara h6 (50 µg/ml). At day 23, supernatants were harvested and stored at -20°C. [^{3}H]-TdR was added and the cells were harvested after 18 hours as described above. The SI was considered positive when the SI > 2.0.

Determination of cytokines

Cytokines in culture supernatants of primary lymphocyte stimulation tests and short-term T cell

lines (IL-10, IL-13, IFN-γ and TNF-α) were measured by ELISA, according to the manufacturer's recommendations (Sanquin, Amsterdam, the Netherlands). The detection limit was 2.3 pg/ml for IL-10, 1.2 pg/ml for IL-13, 3.1 pg/ml for IFN-γ, and 3.1 pg/ml for TNF-α. Cytokines (IL-5, IL-10, IL-13, IFN-γ, TNF-α) were measured in culture supernatants of peptide stimulation assays using the Multiplex Immunoassay, as described previously. [14]

Statistics

Peanut-specific IgE levels, proliferation and cytokine production by PBMC were not normally divided. Hence, values are reported as median, unless otherwise indicated. Differences between groups were calculated with the non-parametric Man Whitney U or Kruskall Walis test. Wilcoxon matched pairs test was used to compare responses of different peanut allergens within individuals. Non-complete pairs were excluded from the paired calculations. Differences were considered significant if the p-value was < 0.05. Statistical analysis was performed using the Statistical Program SPSS (version 12.0, SPSS Inc., 2001, Chicago USA).

Results

Primary T cell response to crude peanut extract (CPE)

Taking all 3 groups into account, enhanced proliferation after initial CPE stimulation was observed in PA, PS and NA subjects. Although overall the proliferation was higher in PA and PS children than in the NA group, the SI for CPE was not different between the 3 groups (Fig. 1A). Production of IL-13, IFN-γ and TNF-α, but not IL-10, was induced upon stimulation with CPE (Fig. 1B-E). The production of IL-13, IFN-γ and TNF-α was significantly higher in the PA and PS group than in the NA group. No significant differences were observed between PA and PS children.

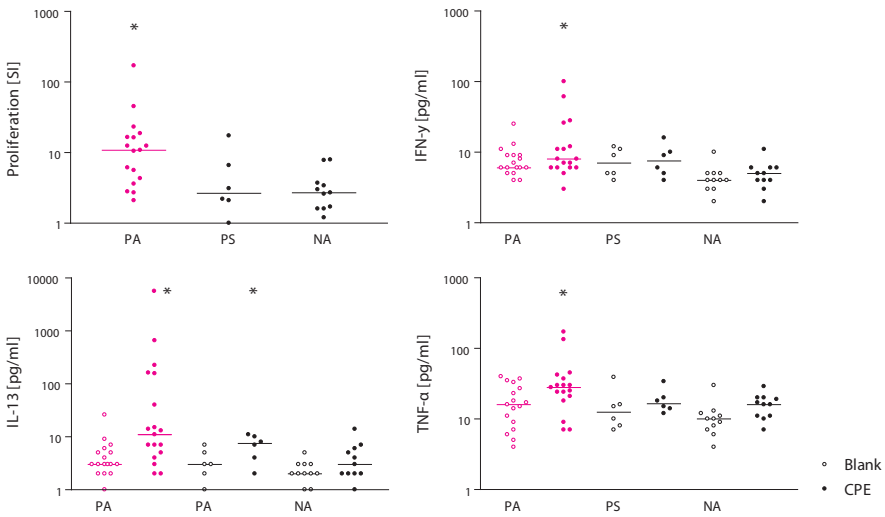
T cell response to CPE in peanut-specific T cell lines

Short-term CPE specific T cell lines were generated to measure the response to major peanut allergens in the different groups. The cell lines were specific for peanut, as could be demonstrated by the unresponsiveness to cow's milk (data not shown).

All PA children had a positive SI to CPE, whereas 5 of 6 PS children responded to CPE, and 7 of 11 NA subjects (Figure 2A). The SI was significantly higher in PA children compared to PS and NA subjects ($p=0.040$ and $p<0.001$). No significant difference was observed between PS and NA subjects. In PA children, IL-13, IFN-γ and TNF-α production increased upon stimulation with CPE (Figure

2B-D). The induction of IL-13 production was also enhanced in PS children, but not in NA subjects. IFN-γ and TNF-α production was not increased in PS and NA subjects. No increase in IL-10 production was observed for any of the groups.

Figure 8.2



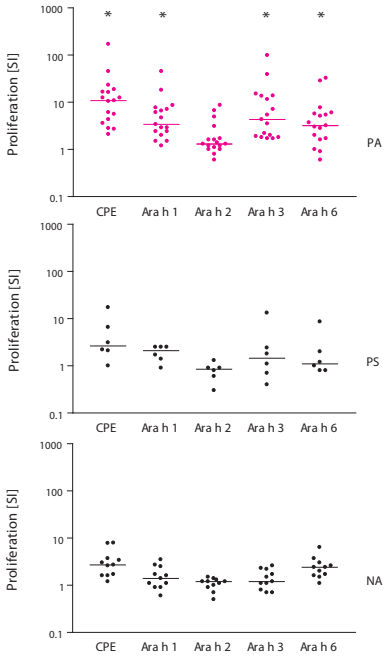
Proliferation (A) is shown as the stimulation index (SI) and cytokine production (B-E) is shown with (○) and without (●) stimulation with CPE for PA (orange), PS and NA groups. Proliferation or cytokine production indicated with (*) is significantly higher than NA subjects. *P* values by Mann Whitney U test; $p<0.05$.

T cell response to peanut allergens Ara h1, Ara h2, Ara h3 and Ara h6

Proliferation to all major allergens was significantly enhanced in the PA group (Fig. 3A). In this group, 14 of 17 (82%) had a positive proliferative response to Ara h1, 4 of 17 (24%) to Ara h2, 12 (71%) to Ara h3 and 12 (71%) to Ara h6. The SI for Ara h3 was highest among the stimulation by major peanut allergens, followed by Ara h1 and Ara h6. The lowest SI was observed for Ara h2. The response to major allergens in the PS and NA group was less pronounced. In the PS group, positive responses were observed to Ara h1 (3/6; 50%), to Ara h3 (2/6; 33%) and to Ara h6 (2/6; 33%). In the NA group, responses were observed to Ara h1 (3/11; 27%), to Ara h3 (3/11; 24%) and Ara h6 (7/11; 64%). None of the PS and NA group had a positive response to Ara h2. The SI of the PA group upon stimulation with Ara h1, Ara h2 and Ara h3 was significantly higher than either PS or NA subjects (Fig 3B and 3C), whereas the SI of Ara h6 was not different between the groups.

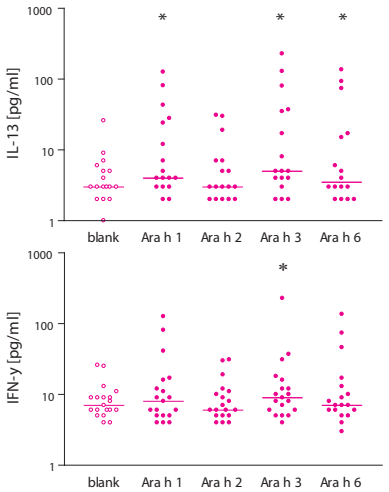
The PA group showed enhanced IL-13 production upon stimulation with Ara h1, Ara h3 and Ara

Figure 8.3



Proliferation (SI) to major peanut allergens in PA subjects (A), PS subjects (B) and NA subjects (C) in short-term peanut-specific T cell lines. Proliferation of PA subjects indicated with (*) is significantly higher than PS or NA subjects. *P* values by Mann Whitney U test; p>0.05.

Figure 8.4



IL-13 (A) and IFN-γ (B) production upon stimulation with major peanut allergens in PA subjects. Differences between major peanut allergens (○) as compared with blank (●) are indicated (*). P values by Wilcoxon matched pairs test: p<0.05.

h6 (Fig. 4A). IL-13 production was in line with proliferation, with highest production in Ara h3 stimulated cells, and lowest in Ara h2 stimulated cells. No significant increase in the production of IFN-γ, TNF-α or IL-10 was observed (Fig. 4B). The PS and NA subjects did not show an increase in production of any cytokine.

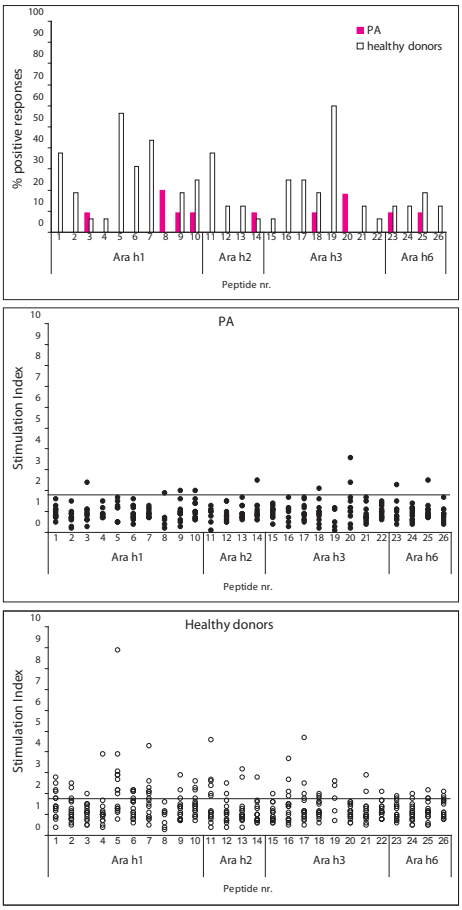
T cell response to a selection of peanut peptides

In 11 PA subjects and 16 HC, responses of fresh PBMC were measured towards 26 pan-HLA-DR binding potential T cell epitopes (Figure 5A). In only 3/ 11 PA subjects, positive responses (SI > 1.8) could be detected, with 10 responses out of a total of 263 responses measured (3.8%). Of these responses, 4/101 were directed to peptides from Ara h1 (4.0%), 1/40 towards Ara h2 (2.5%), 3/78 responses towards Ara h3 (3.9%), and 2/44 towards Ara h6 (4.5%) (Figure 5B). In 15/16 HC donors, 77 positive responses were detected, out of a total of 397 (19.4%). This fraction of positive responses

was significantly higher than in the PA subjects ($p<0.001$). Of these responses, 39/152 (25.7%) were directed to Ara h1 peptides, 11/64 to Ara h2 (17.2%), 18/177 towards Ara h3 (15.4%), and 9/64 towards Ara h6 (14.1%) (Figure 5C). The frequency of positive responses was not correlated with the predicted binding score of the peptide (data not shown).

Cytokine levels were measured in culture supernatant by using the Multiplex Immunoassay. Upon stimulation with peptides, no significant induction of cytokines was observed (data not shown). As a control, stimulation with CPE was included. Production of IL-13, and here also of IL-5, were significantly higher in peanut-allergic than in healthy donors. IFN-γ and TNF-α were induced in both groups, but levels were not significantly different. No production of IL-10 was observed (data not shown).

Figure 8.5



Proliferation upon peanut peptide stimulation.
PBMC of PA (n=11) and HC donors (n=16) were stimulated with pan-HLA-DR binding peptides for 6 days. Responses were considered positive when SI>1.8. For each peptide, the % positive responses is shown (A), as well as the stimulation index of individual PA (B) and HC donors (C).

Discussion

The T cell response to peanut in PA children was characterized by production of IL-13 and IFN-γ, both in the primary assay and in T cell lines. Looking at stimulation of peanut-specific T cell lines with purified major peanut allergens, PA children showed enhanced proliferation associated with higher production of IL-13 to each of the major allergens, in contrast to PS children and NA subjects. T cell proliferation and IL-13 production was remarkably low after stimulation with Ara h2 compared to stimulation with other peanut allergens. In search of immunodominant T cell epitopes in the major allergens, it appeared that PA subjects had a remarkably reduced response compared to control subjects.

Investigation of the T cell response in PA children demonstrated strong proliferation to peanut, which has been reported before. [4] Besides an enhanced production of the Th2 cytokine IL-13, also a significant increase in IFN-γ production was observed after stimulation with peanut. In a previous study, no IFN-γ response was noticed. [5] However, in a mouse model using the same peanut extract as in the present study, also both Th1 and Th2 cytokines were induced. [15] In children with persistent cow's milk allergy a similar induction in IL-13 and IFN-γ production by T cells was observed upon stimulation with cow's milk. [16] This suggests that in persistent food allergy both Th1 and Th2 cytokines play a role.

Comparison of PA children with PS children showed that there were no significant differences in the primary response to crude peanut, which is in line with a previous report. [5] Looking at the T cell responses to CPE and major peanut-allergens in peanut-specific T cell lines, the proliferation and cytokine production of PA children was enhanced compared to proliferation and IL-13 production of PS children. Apparently, the T cells from PA subjects maintain a higher activation level during the generation of short-term T cell lines compared to T cells from PS subjects. This finding is in line with the long-term activation status of T cell clones from cow's milk allergic subjects in a previous study, characterized by much higher levels of CD25 and CD30 expression compared to tolerant subjects. [17] Still, PS subjects had an increased production of IL-13 to CPE as compared with NA subjects. The ability of inducing Th2 responses to peanut underlines the fact that these tolerant subjects had detectable peanut-specific IgE.

In another study, cow's milk tolerant atopic children were characterized by high production of IL-10 by cow's milk specific T cells. [16] It was suggested that IL-10 in these children may be involved in the maintenance of tolerance in an atopic environment. In a recent study, the production of IL-10 has been associated with high levels of allergen-specific IgG4 to cow's milk in tolerant atopic children.

Table I. Characteristics of pan-HLA-DR binding peptides

Peanut allergen	Accession code	Peptide nr.	Position	Core sequence
Cut-off Sette				
Ara h1	P43238	1	15	LVLASVSAT
		2	20	VSATHAKSS
		3	158	WGTPGSHVR
		4	214	IVQIEAKPN
		5	283	LRVAKISMP
		6	428	LQDLDMMLT
		7	438	IKEGALMLP
		8	448	FNSKAMVIV
		9	510	FIMPAAHPV
		10	582	FVSARPQSQ
Ara h2	Q8GV20	11	14	LLAAHASAR
		12	37	LERANLRPC
		13	124	IMENQSDRL
		14	141	FKRELRNLP
Ara h3	Q8LKN1	15	103	LIFPGCPST
	Q5I6T2	16	158	VPTGVAFWM
		17	266	EFLAQAFQV
		18	374	IYNPQAGSL
		19	388	LQLNLLILR
		20	474	YVAFKTDSR
		21	517	LKNNNPFKF
		22	525	FFVPPSQQS
Ara h6	Q9SQG5	23	34	HIMQRIMGE
		24	49	YNFGSTRSS
		25	84	IMENQCDGL
		26	101	FKRELMNLP

Peanut allergen	Accession code	Score DR1	Score DR4	Score DR7	Score above cut-off
Cut-off Sette		0.183	0.734	1.749	
Ara h1	P43238	15.2	55	173	DR 1, 4, 7
		4.4	1.9	49.5	DR 1, 4, 7
		4.8	28.1	7.9	DR 1, 4, 7
		14.2	1.1	2.4	DR 1, 4, 7
		2.8	2.7	25.1	DR 1, 4, 7
		37.5	0.6	4.3	DR 1, 7
		124.7	4	2.5	DR 1, 4, 7
		10.8	0.6	10.6	DR 1, 7
		5505.6	149.9	97.5	DR 1, 4, 7
		4.2	3	5.2	DR 1, 4, 7
Ara h2	Q8GV20	68.7	0.9	166.3	DR 1, 4, 7
		0.24	0.08	0.27	DR 1, 7
		0.09	7.1	0.39	DR 4
		0.15	0.02	0.07	none
Ara h3	Q8LKN1	12.4	11.5	2	DR 1, 4, 7
	Q5I6T2	9.4	1	5.4	DR 1, 4, 7
		4.1	0.3	5.7	DR 1, 7
		3.6	2.4	2.2	DR 1, 4, 7
		18.1	2	3.5	DR 1, 4, 7
		0.2	4.6	2.6	DR 1, 4, 7
		3.4	6.1	12.6	DR 1, 4, 7
		137.3	10.7	3.4	DR 1, 4, 7
Ara h6	Q9SQG5	0.6	0.04	2.1	DR 1, 7
		0.59	0.75	0.13	DR 1, 4
		0.02	8.8	0.34	DR 4
		39.8	1.15	8.4	DR 1, 4, 7

[18] A similar association between IL-10 and IgG4 has been reported before during the induction of tolerance by immunotherapy. [19] However, IL-10 production was not detected in tolerant peanut-sensitized subjects from this study. Accordingly, also low binding of IgG4 to major peanut allergens was observed (unpublished data). Subjects from the present study were sensitized to peanut but never had an allergic reaction to peanut, whereas the cow's milk tolerant atopic subjects were never sensitized to cow's milk. [16] Because no differences in Th1 cytokine production and no IL-10 production was observed in our study, apparently, Th1 responses or regulatory responses to peanut and major peanut allergens may not be the compensatory mechanisms for peanut-specific IgE production in our tolerant population.

This is the first report of T cell responses to major peanut allergens in peanut-allergic subjects. All PA children showed a response to one or more of the major allergens dominated by the production of IL-13, as was expected for allergic subjects. [3;5] The T cell response to Ara h2 was remarkably low, with significant proliferation in only 4 of the 17 peanut-allergic children. Previous studies investigating the B cell responses to major allergens showed that Ara h2 and Ara h6 were recognized by IgE of all peanut-allergic individuals. [6;20;21] In contrast to Ara h2, Ara h6, which is 70% homologous with Ara h2, was able to induce a significant T cell response. The discrepancy between the B and T cell responses for Ara h2 can not be explained by differences in the allergen source because the same stock of purified allergens was used. A similar finding was reported for cow's milk: although IgE was directed predominantly towards αs1-casein, the T cell response to αs1-casein seemed impaired compared to the T cell response to other cow's milk allergens (unpublished data). Because the exposure to all peanut allergens *in vivo* coincides, the T cells responding to other allergens and compounds in peanut than Ara h2 may provide bystander help for the IgE response to Ara h2. The glycan structures on Ara h1 for instance can act as a ligand for dendritic cells, thereby creating a Th2-skewing environment which may skew the T cell response towards other peanut allergens as well. [22]

A study of T cell responses at the level of epitopes may help to elucidate the molecular mechanisms of tolerance or peanut allergy. In order to investigate potential immunodominant peptides in the peanut allergens, candidate epitopes were selected using a method previously successfully used to identify T-cell epitopes in microbial antigens or auto-antigens (heat shock proteins). [10;23;24] Several peanut peptides, derived from different peanut allergens, were able to induce proliferation. In contrast to what we expected, control subjects proliferated more to peptides than PA subjects. The cytokine production was too low to determine differences in the cytokine profile. A proposed explanation is that these PA subjects have been on elimination diets for a prolonged period. Perhaps

peanut-specific T cells have resided in peripheral lymph nodes resulting in low frequencies in the periphery during elimination of peanut, whereas activated T cells re-enter the circulation after exposure to peanut and therefore more peripheral T cell reactivity towards peanut can be observed. Indeed, in a pilot experiment in 2 peanut-allergic children, a T cell response to peanut peptides was not observed before DBPCFC, but was detected after exposure to peanut during DBPCFC (data not shown). Determination of T cell epitopes is a relevant issue for the development of novel immunotherapeutic regimens, and the use of a computer algorithm can facilitate this research.

Conclusion

The T cell response in peanut-allergic children was characterized by both IL-13 and IFN-γ after stimulation with CPE. Although no differences between peanut-allergic and non-allergic peanut-sensitized children was observed after primary stimulation with CPE, in CPE-specific polyclonal T cell lines only peanut-allergic children showed enhanced Th2 responses to Ara h1, Ara h3 and Ara h6, but not to Ara h2. A selection of peanut peptides based on a computer algorithm, derived from the different peanut allergens, was able to induce T cell responses as well. Further research on T cell responses to peanut and peanut peptides is needed in order to better understand which peptides are immunodominant in peanut allergy.

Acknowledgements

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Probiotics have a different immunomodulatory potential in vitro versus ex vivo upon oral administration in children with food allergy

Abstract

Background: Previous studies suggest that administration of probiotics *in vitro* can stimulate regulatory and Th1 immune responses. We studied both the *in vitro* immunological effects of probiotics and the *ex vivo* immunological effects after oral administration of probiotics in children with food allergy, a Th2-mediated disease.

Methods: Thirteen children were enrolled. Probiotics (n=7) or placebo (n=6) were orally administered during 3 months. At t=0, t=1 and t=3 months, PBMC were stimulated with crude peanut extract, anti-CD3, or anti-CD40 and IL-4 in the presence (*in vitro* response) or absence (*ex vivo* response) of probiotics. Proliferation and production of IFN-γ, IL-5, IL-13, IL-10, TNF-α , IL-6 and IgE were analyzed. Sensitization to peanut, cow's milk and hen's egg was determined before and after treatment.

Results: The *in vitro* addition of probiotics to PBMC cultures resulted in enhanced proliferation and production of IFN-γ, IL-10 and TNF-α. After oral treatment, the proliferation in the presence of probiotics increased, whereas *in vitro* IgE production decreased in the probiotics group compared to baseline. The *ex vivo* production of IL-10, TNF-α and IL-6 tended to decrease. Th1 and Th2 cytokines were not altered. Sensitization remained unchanged.

Conclusion: Probiotics enhanced the production of Th1 and regulatory cytokines *in vitro*. Oral administration of the probiotics resulted in a slightly decreased *ex vivo* production of IL-10, TNF-α and IL-6. This indicates that probiotics have a different potential to modulate the immune response *in vitro* versus *ex vivo*.

Introduction

In recent years, atopic diseases in westernized countries have become an increasing health problem. [1] The 'Hygiene' hypothesis explains this increase by a diminished exposure to microbes in the ecosystem. [2] A reduced microbial stimulation during infancy and childhood may result in an inadequate postnatal maturation of the immune system leading to a suboptimal balance between Th1 and Th2 immunity. Studies have demonstrated that infants who develop allergy have a different composition of the gut flora. [3;4] Administration of lactic acid bacteria in adequate amounts, so- called probiotics, may compensate for the loss of immunological stimuli by microorganisms and confer a health benefit. [5] In *in vitro* PBMC models, probiotic strains were able to increase the production of regulatory cytokine IL-10 and inflammatory cytokines IL-12 and TNF-α by monocytes and dendritic cells, and enhance IFN-γ production by T-cells. [6-8] According to these *in vitro* studies that show a potential of probiotics to divert the immune response from a Th2 profile, probiotics may be beneficial in atopic subjects. [9;10] Modulating the typical Th2 response associated with food allergy [11] may result in a decreased specific IgE production by B-cells. The limitation of studying the effect of probiotics *in vitro* is the extrapolation of the results to *in vivo* benefits. Few studies have investigated the immunological effects on mitogen-induced PBMC responses after oral administration of probiotics in children with food allergy. [12-14] The effect on allergen-specific immune responses has not been studied before.

This study was set up to investigate whether *in vitro* immunological effects of probiotics on allergen-specific responses are reflected in changes in *ex vivo* responses after oral treatment. First, we investigated the *in vitro* response of PBMC to a mixture of probiotics in young children with food allergy. Then, these children were treated with probiotics or with placebo. Proliferation and cytokine production by PBMC after allergen-specific or anti-CD3 stimulation at 1 and 3 months were compared to the outcome before treatment. Secondary to the immunological response by PBMC, the allergen-specific sensitization to food was monitored before and after treatment.

Methods

Patients

Thirteen children (3 female and 10 male) aged 0.5 – 2.8 years were recruited from the Department of Pediatric Dermatology and Allergology, Wilhelmina Children's Hospital at the University Medical Center Utrecht, The Netherlands (Table I). They were sensitized to at least 2 common food allergens

(peanut, hen's egg or cow's milk) and had a convincing history of food allergy. Elimination of the culprit food was advised at the outpatient clinic before the start of the study. All children had atopic dermatitis before enrolment in the study, which was treated accordingly. Six subjects had symptoms of bronchial hyperreactivity and wheezing and 2 subjects had rhinoconjunctivitis, diagnosed by the pediatrician. During the study, all subjects continued their usual medication. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht. All parents gave written informed consent before enrolment of their children in the study.

Table I. Patient characteristics at t=0 for the probiotics group (n=7) and the placebo group (n=6).

	Probiotics* (n=7)	Placebo* (n=6)
Patient demographics		
age (years)	1.4 (0.5-2.1)	2.0 (0.7-2.8)
boys	5/7	5/6
Atopic diseases		
Atopic dermatitis	7/7	6/6
SCORAD	23 (0-37)	19 (8-36)
bronchial hyperreactivity	3/7	3/6
rhinoconjunctivitis	0/7	2/6
food allergy	7/7	6/6
Sensitization†		
IgE total	476 (101-1003)	615 (231-1299)
IgE peanut	29 (0-100)	41 (0.9-90)
IgE hen's egg	28 (0.3-100)	38 (0.7-65)
IgE cow's milk	15 (0-86)	39 (0.9-68)
SPT peanut	11 (0-14)	12 (7-18)
SPT hen's egg	7.9 (7-9.5)	7.5 (4-9.5)
SPT cow's milk	5.0 (0-9.5)	7.3 (0-14)

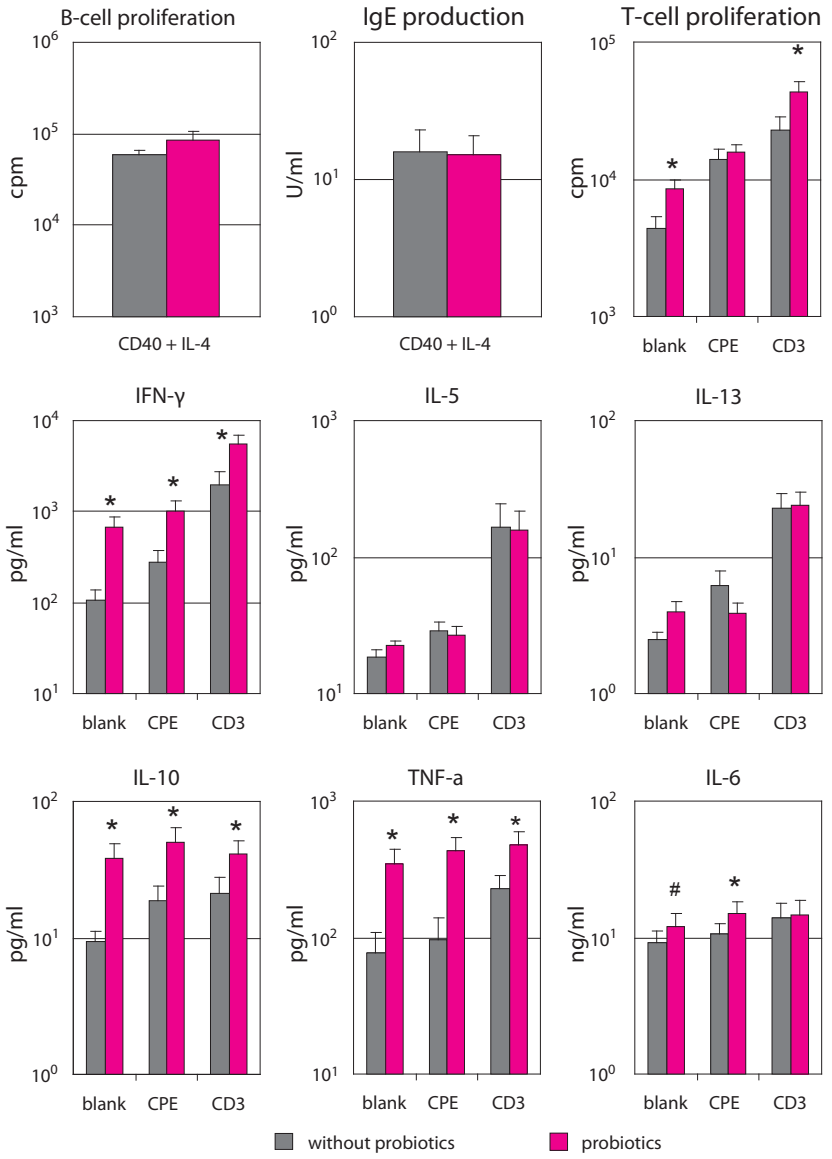
*Results are displayed as mean (range) or number/group

† Specific IgE levels in kU/L and SPT in mm

Probiotics

Probiotics or placebo was randomly and double-blind assigned to the patients. The probiotics contained a mixture of probiotic strains (*Lactobacillus (L.) acidophilus* W55, *L. casei* W56, *L. salivarius* W57, *Lactococcus (Lc.) lactis* W58, *Bifidobacterium (B.) infantis* W52, *B. lactis* W18 *and B. longum* W51, Winclove Bio Industries, Amsterdam, the Netherlands). These individual strains were shown to induce the production of Th1 and regulatory/inflammatory cytokines by PBMC of

Figure 9.1



At start of the study, t=0 months, the *in vitro* effect of probiotics on proliferation and cytokine production was investigated. PBMC were left unstimulated (blank) or were stimulated with CPE or CD3, in the absence (grey bars) or presence (orange bars) of probiotics. Data are shown as mean + standard error of the mean of all subjects (n=13). *P* values by Wilcoxon matched pairs test: * $p<0.05$; # $p=0.05$.

healthy adults. [15] No LPS was detected in the mixture. The freeze-dried probiotics were masked in a powder consisting of rice starch and maltodextrine. A dose of 10^9 colony-forming units was given orally once daily for 3 months. Placebo portions consisted of rice starch and maltodextrine only, and were given at the same frequency. The powder was dissolved in hand-warm water or infant formula before administration, to rehydrate the probiotic bacteria.

PBMC cultures

Venous heparinized blood was collected at the start of the treatment period, after 1 month and after 3 months of treatment. PBMC were isolated using Ficoll-Isopaque centrifugation (Amersham Biosciences, Uppsala, Sweden). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, NY, USA) supplemented with 2% pooled human recalcified AB-plasma and 5% Yssel's medium [16] supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamin (1mM) (Gibco).

T cell proliferation tests

Lymphocyte stimulation tests (LSTs) were performed in quadruplicate in 96-well flat-bottom plates (Greiner, Frickenhausen, Germany). Each well contained $1*10^5$ cells, and cells were either left unstimulated, or were stimulated with crude peanut extract (CPE, 50 µg/ml, kindly provided by dr. S.J. Koppelman, TNO Quality of Life, Zeist, The Netherlands) or soluble anti-CD3 (2 µg/ml, Sanquin, Amsterdam, the Netherlands), in the presence or absence of probiotics ($1*10^5$ colony-forming units /well, previously determined to be optimal). Stimulation with CPE was chosen as a model for the immunological response to food allergens. Freeze-dried probiotics were dissolved in PBS and added directly to the co-culture.

Proliferation was measured after 7 days by [^{3}H]-TdR incorporation (1 µCi/well; Amersham, Aylesbury, UK), which was added during the last 18 hours of culturing. Proliferative responses are expressed as the mean [^{3}H]-TdR incorporation (cpm) of quadruplicates. Prior to the addition of [^{3}H]-TdR, supernatant was collected and stored at -20˚C.

Cytokine ELISA

The production of cytokines was measured in the supernatant of PBMC cultures after CPE or anti-CD3 stimulation, or of unstimulated cells, in the presence or absence of probiotics. ELISAs specific for IL-5 (Endogen, Woburn, USA), and for IL-6, IL-10, IL-13, IFN-γ and TNF-α (Sanquin, Amsterdam, the Netherlands) were used according to the manufacturer's instructions. The detection

limits were 10 pg/ml for IL-5, 1.3 pg/ml for IL-6, 2.3 pg/ml for IL-10, 1.2 pg/ml for IL-13, 3.1 pg/ml for IFN- γ, and 3.1 pg/ml for TNF-α.

B-cell proliferation

PBMC were stimulated with anti-CD40 (2.5 μg/ml, clone 5C3, BD Pharmingen, San Diego, USA) and IL-4 (200 U/ml, a kind gift from Novartis Research Institute, Vienna, Austria) to enhance B-cell activation in the presence or absence of probiotics. After 14 days of culturing, supernatants were harvested to determine the production of IgE by ELISA. [^{3}H]-TdR was added (1 μCi/well) during the last 18 hours of culturing, and proliferation was measured.

IgE ELISA

To measure IgE in culture supernatants, Nunc maxisorp plates were coated for 1 h at RT with goat-anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA) in PBS, and blocked with PBS/5%FCS. A serum sample with known IgE concentration was used as standard (highest concentration of standard curve 32 U/ml, detection limit 0.5 U/ml). Samples were incubated for 1 h at RT. Biotin-conjugated mouse-anti-human IgE (BD Pharmingen) was used as detection antibody, and was incubated for 1 h at RT. The standard, culture supernatants, and detection antibody were diluted in PBS/5%FCS/0.05%Tween. HRP-conjugated streptavidine (Sanquin) was incubated for 30 min at RT. Substrate (KPL, Gaithersburg, MD, USA) was added, and the reaction was stopped with 1M H_3PO_4. The OD was measured at 450 nm.

Allergic sensitization

Patients visited our outpatient clinic monthly during the treatment. At the first and last visit, specific IgE for peanut, hen's egg and cow's milk were measured using CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden) in accordance with manufacturer's instructions. Skin prick tests (SPT) were performed on the patients' back with standard lancets and commercial extracts of peanut, hen's egg and cow's milk (ALK- ABELLÓ, Nieuwegein, the Netherlands). 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. The wheal diameter (mm) was measured after 15 minutes.

Statistics

Values are reported as mean, unless otherwise indicated. Proliferation, cytokine production, and IgE production by PBMC were not normally divided. Also specific serum IgE levels and SPT

wheal sizes were not normally divided. Hence, differences in time were calculated with the non-parametric Wilcoxon matched pairs test, and were considered significant if the p-value was ≤ 0.05. One child dropped out one month before the end of the study on request of the parents, because the child developed otitis media and received antibiotics (placebo group). Data of this child retrieved from the first 2 months were used in further calculations. From 5 time points, we were not able to retrieve enough PBMC to measure all variables, due to the strict regulation on blood sampling for research purposes in children. Non-complete pairs were excluded from the paired calculations. Statistical analysis was performed using the Statistical Program SPSS (version 12.0, SPSS Inc., 2001, Chicago USA).

Results

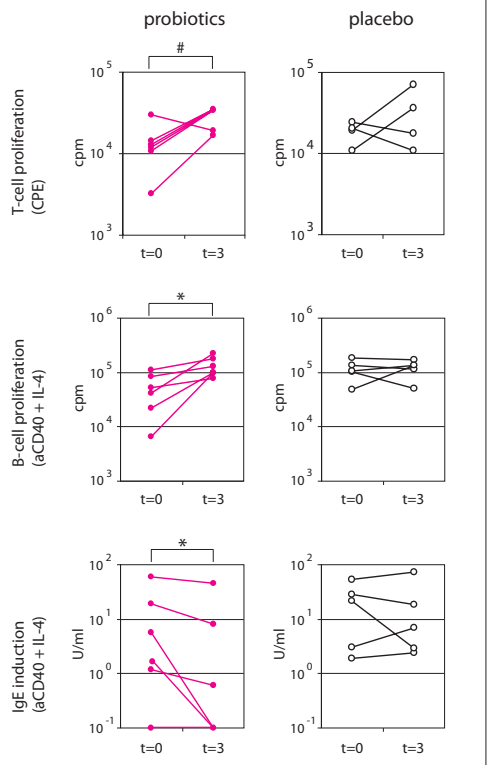
The in vitro effect of probiotics

Thirteen children were included. The patient characteristics before start of the treatment, at t=0 months, were comparable for the probiotics group (n=7) and the placebo group (n=6) (Table I). The *in vitro* effect of the probiotics on the response of PBMC was investigated by adding the probiotics to the PBMC cultures. Data were pooled for all 13 children, and are shown in Figure 1. T-cell proliferation was significantly enhanced by the probiotics in the unstimulated and anti-CD3 stimulated cultures, but not in the CPE-stimulated cultures. The production of IFN-γ, IL-10 and TNF-α was strongly enhanced. IL-6 production was slightly enhanced in the CPE-stimulated culture. The cytokines IL-5 and IL-13 were not influenced. After stimulating the cells with anti-CD40 and IL-4, B-cell proliferation and IgE production in the supernatant were not altered. When analyzed separately, these results at t=0 were similar for the probiotics group and the placebo group. So, *in vitro*, probiotics stimulated the T-cell response towards a Th1/regulatory response, but did not affect the B-cell response.

The in vitro effect of probiotics after oral treatment

To determine the effect of oral treatment with probiotics on the *in vitro* immune response to probiotics, PBMC collected after treatment were stimulated in the presence of probiotics. As at t=0, T-cell proliferation and production of IFN-γ, IL-10 and TNF-α at t=3 months were strongly enhanced in all cultures, both in the probiotics-treated and the placebo group. In the probiotics group, CPE-stimulated, but not unstimulated and anti-CD3 stimulated T-cells proliferated stronger in the presence of probiotics *in vitro* after 3 months of treatment than at t=0 (Figure 2). This was not

Figure 9.2



The *ex vivo* effect of the mixture of probiotics on proliferation and cytokine production induced by CPE at t=0, t=1 and t=3 months. Results of all individual patients are shown for the probiotics group (closed orange symbols) and the placebo group (open symbols). Calculations were based on Wilcoxon matched pairs test: # p=0.063.

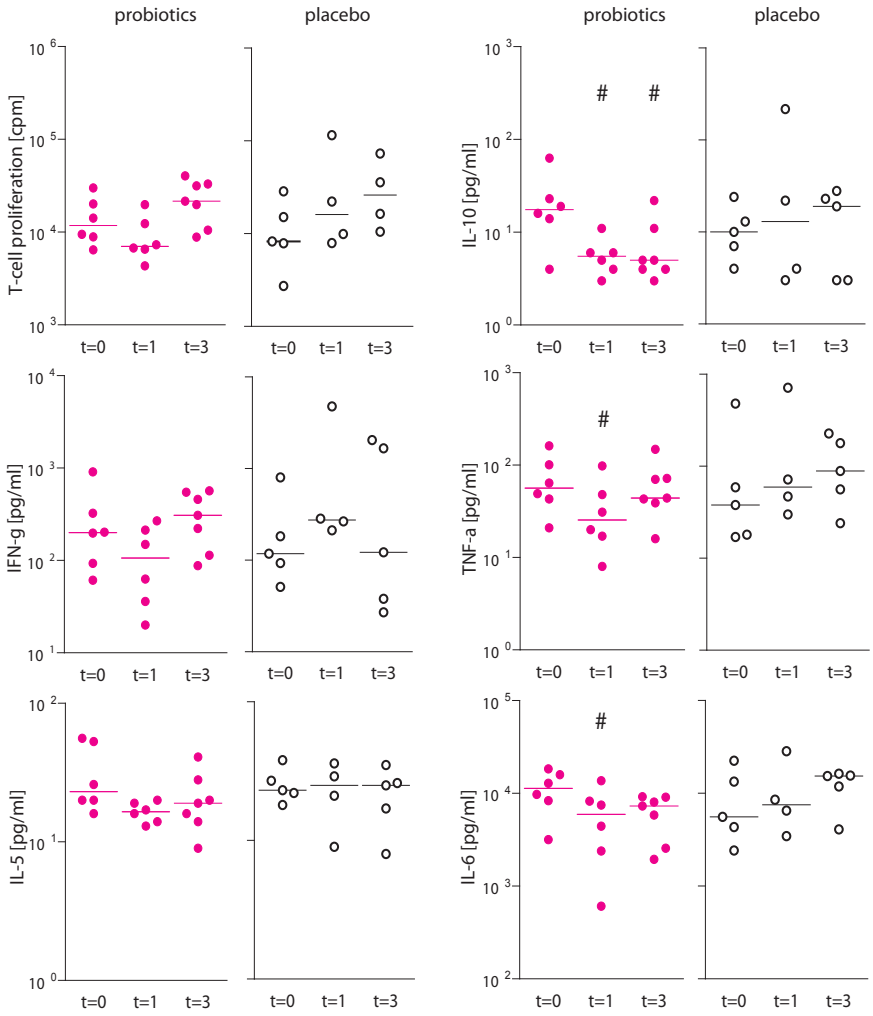
observed in the placebo group. No difference in cytokine production was observed (data not shown). B-cell proliferation in the anti-CD40 and IL-4 stimulated cell cultures in the presence of probiotics was significantly enhanced in the probiotics group at t=3 months compared to t=0. The IgE production *in vitro* significantly decreased in this group (Figure 2). Overall, oral treatment with probiotics, but not with placebo, enhanced the proliferative response to probiotics *in vitro* after 3 months compared to t=0.

The ex vivo effect after oral treatment with probiotics

The effect of oral treatment with probiotics on the *ex vivo* food allergen-specific or the overall immunological response of PBMC without addition of probiotics *in vitro* was investigated. Therefore, PBMC before and 1 and 3 months after treatment were stimulated with CPE or anti-CD3, or with anti-CD40 and IL-4, in the absence of probiotics. The results for CPE stimulation at t=0, 1 and 3 months are shown in Figure 3. At t=0, proliferation and cytokine production were comparable

between both groups. T-cell proliferation with CPE stimulation seemed slightly enhanced both in the probiotics and in the placebo group after 1 and 3 months, but this increase was not significant. Th1 (IFN-γ) and Th2 cytokine (IL-5, IL-13) production upon CPE or anti-CD3 stimulation was unaffected in both groups. In the probiotics group, the production of the regulatory cytokine IL-10 both in unstimulated and CPE-stimulated cultures tended to be reduced after 1 and 3 months (p=0.063). In

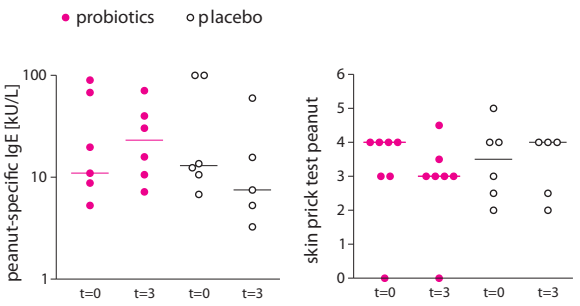
Figure 9.3



T and B-cell proliferation and IgE induction after addition of probiotics *in vitro* at t=3 months compared to t=0 months. Paired results of individual patients are shown for the probiotics group (closed orange symbols) and the placebo group (open symbols). Samples without detectable IgE (below the detection limit of 0.5 U/ml) are shown as 0.1 U/ml. *P* values by Wilcoxon matched pairs test: * p<0.05; # p=0.063.

anti-CD3 stimulated cultures no significant decrease was observed (not shown). The production of inflammatory cytokines TNF-α and IL-6 in unstimulated, CPE- and CD3-stimulated PBMC tended to decrease in the probiotics group after 1 month (p=0.063). After 3 months, the production of TNF-α and IL-6 returned to levels comparable to t=0. The IgE production after anti-CD40 and IL-4 stimulation was unaffected by oral administration of the mixture of probiotics (data not shown). Summarizing, oral treatment with probiotics resulted in a temporary slight decrease of inflammatory and regulatory cytokines, whereas the production of Th1 and Th2 cytokines was unaffected.

Figure 9.4



Specific serum IgE levels (kU/L) and SPT wheals (mm) for peanut at t=0 and t=3 months. Individual data and median values are shown for the probiotics group (closed orange symbols) and the placebo group (open symbols).

The effect of oral treatment with probiotics on allergic sensitization

Specific IgE levels for peanut, hen's egg and cow's milk did not change after 3 months compared to t=0 months in both groups. SPT wheal sizes did not change either (shown for peanut in Figure 4).

Discussion

In children with food allergy, addition of probiotics to PBMC cultures *in vitro* showed increased T -cell proliferation with enhanced production of Th1 and regulatory cytokines. Oral administration of probiotics tended to result in a decreased *ex vivo* production of IL-10, TNF-α and IL-6 by PBMC. This result was most pronounced after 1 month of treatment, and faded after 3 months for TNF-α and IL-6. Th1 and Th2 cytokines were not altered. After 3 months of oral treatment, in the presence of probiotics *in vitro*, an increase in T- and B-cell proliferation and a reduction of IgE production was observed. Sensitization to food allergens was not altered.

The *in vitro* addition of the probiotics to the PBMC cultures resulted in enhanced T-cell proliferation, and increased production of IFN-γ, IL-10, TNF-α, and to a lesser extent IL-6. This finding confirmed

the data obtained from the individual strains. [15] Other *in vitro* studies also reported a strong induction of regulatory or Th1 cytokines. [6-8;17]

After 3 months of oral treatment with probiotics, in contrast to placebo, both T and B-cells proliferated significantly more after addition of probiotics *in vitro*, when compared to t=0. It could be speculated that the cells were primed by the exposure to probiotics within the intestinal mucosa and entered the peripheral blood. PBMC containing these supposed memory cells may respond stronger upon triggering by probiotics *in vitro*.

In contrast to the *in vitro* effects of probiotics, the *ex vivo* effect of oral administration of the same probiotics was an overall clear tendency towards decreased production of IL-10, TNF-α and IL-6. Only few studies have investigated cytokine profiles in atopic children after oral treatment with probiotics. Rosenfeldt et al. [18] did not find any change in cytokine production. Pohjavuori et al. [13] and Prescott et al. [14] reported an increase of IFN-γ, only after CD3/CD28 or mitogenic, but not allergen-specific stimulation. None of these studies reported the effect of the used probiotics per se on the PBMC response *in vitro*. Our data demonstrate that the direct contact between probiotics and PBMC *in vitro* does not reflect the situation when individuals are exposed to the same probiotics via the intestinal mucosa.

The decreased production of IL-10, TNF-α and IL-6 seemed mostly independent of the stimulation (blanco, CPE or anti-CD3), suggesting that the overall immune response was affected rather than the allergen-specific response. The decreased cytokines can all be produced by monocytes, indicating that the *ex vivo* effect of probiotics may be related to monocytes. It is not clear from the current study how monocytes can be affected after oral administration of probiotics. An explanation could be the promotion of gut barrier functions by probiotics. [9;19;20] A diminished bacterial translocation may lead to less activated monocytes in the circulation, resulting in a decreased production of monocyte-derived cytokines.

The decreased cytokine production observed *ex vivo* was temporary. The reduction after 1 month was partially reversed after 3 months. Other studies reporting an immunological effect of probiotics *in vivo* [12;13;21] used a 4-week administration protocol. The probiotics, when daily added, may acquire a 'tolerated' niche in the intestinal system after a certain period. [22] Despite the different microbial stimuli of the strains present in the mixture used in our study, this 'tolerance' mechanism may be the reason that the observed immunological effects were partially reversed in our system after 3 months. In order to modulate the immune system with probiotics, it may be necessary to change the strains or interrupt the administration regularly. In this way, the immune system may remain challenged and the effect of probiotics may be prolonged. [22]

The potential to divert the immune response from a Th2 profile *in vitro* suggests that children with food allergy might benefit from probiotics. The IgE production by the proliferating B-cells *in vitro*, in the presence of probiotics, was diminished after 3 months in the children receiving probiotics. This may indicate that administration of probiotics *in vivo* has the potential to induce changes in the B-cell response, and perhaps also the underlying Th2 response. However, the effect was not strong enough to be detected in an *ex vivo* PBMC model, as the production of Th1 and Th2 cytokines after allergenic stimulation with CPE remained unaffected throughout the treatment period. Also, no changes in sensitization were detected. Our study group had strong sensitization for multiple food allergens, probably associated with strongly skewed Th2 responses, which may be difficult to divert. In children with a less Th2-skewed profile, probiotics may have a better effect. The study of Kalliomaki et al. [23] has suggested that probiotics could have a role in the prevention of AD in newborns, whose intestinal and immunological system is still immature. Modulation by probiotics may be easier attained in these immature systems.

Conclusion

In young children with food allergy, the administration of a mixture of probiotics to PBMC cultures *in vitro* showed a promising elevation of Th1 cytokines and regulatory IL-10. Oral administration of the same probiotics resulted in a transient *ex vivo* immunological effect tending towards diminished production of IL-10, TNF-α and IL-6. Although the data suggest that probiotics might have some potential to modulate the immune response *in vivo,* this study shows that caution with extrapolating *in vitro* results is warranted in studies with probiotics.

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10

General discussion

An important role for DBPCFC in hazelnut and peanut allergy in children

Hazelnut and peanut allergy are characterized by sensitization; i.e., allergen-specific IgE, which can be detected in the blood of individuals. [1;2] However, not all children with sensitization to hazelnut and peanut will develop allergic reactions. [3] A history of previous allergic reactions to hazelnut or peanut is of relevance for a good diagnosis. Unfortunately, in 86% of the hazelnut-sensitized children and 37% of the peanut-sensitized children in our study the history was hampered by the fact that parents did not recall previous ingestion of the culprit food. This high prevalence of unknown previous exposure is in line with other studies in children, whereas adults usually present with a history of previous allergic reactions. [4]

We showed that 46% of children with sensitization to hazelnut and 81% of children with sensitization to peanut developed allergic reactions after DBPCFC. As expected, children with a history of previous allergic reactions were more likely to develop allergic reactions during challenge: 75% for hazelnut and 94% for peanut. In the group without previous known intake these percentages were only 38% and 60%, respectively. In other studies it was reported that the percentage of children with sensitization to peanut that developed allergic symptoms during challenge varied between 35% and 61%. [5-7] For hazelnut allergy in pediatric populations this is not known. Apparently the percentage of children with peanut allergy in our study is higher than in other studies. Differences between our study and previous studies may be related to the study population. Unlike others, we included sensitized children without restrictions with respect to the level of sensitization, as determined by specific IgE and SPT. [5] Levels above a defined cut-off have previously been related to the outcome of DBPCFC, with higher levels/reactivity associated with a greater likelihood of positive reaction to challenge. [3;6;8-10] Because cut-off levels depend on the population under study, the value of such cut-off levels remains limited. The fact that we included children with high levels of sensitization, together with the fact that they were referred to our tertiary clinic specialized in food allergy, has most probably increased the selection of children with peanut and/or hazelnut allergy.

Despite the selection of this potentially allergic population, the diagnosis of hazelnut allergy was rejected in at least 54% and the diagnosis of peanut allergy in 19%. These percentages were higher in the group that had no history of previous exposure compared to the group that reported previous allergic reactions. This indicates that history, if present, can improve diagnostic accuracy, although even in this group the diagnosis was rejected in a subset of sensitized children. Taking into account

that this disease will usually persist throughout life, [11-13] a correct diagnosis is mandatory in order to prevent unnecessary dietary restrictions. We showed that DBPCFC is a valuable tool to diagnose hazelnut and peanut allergy. [14] Additionally, establishing a correct diagnosis appeared to have a beneficial effect on the levels of anxiety experienced by the parents of these children. We showed that before DBPCFC, parents were relatively anxious about allergic reactions. High levels of anxiety can be associated with impairments in daily life, which is in line with the lower quality of life in children with (presumed) food allergy and their parents. [15-18] After DBPCFC, the anxiety was significantly reduced not only in the group with negative outcome, but even in the group in which the diagnosis was confirmed.

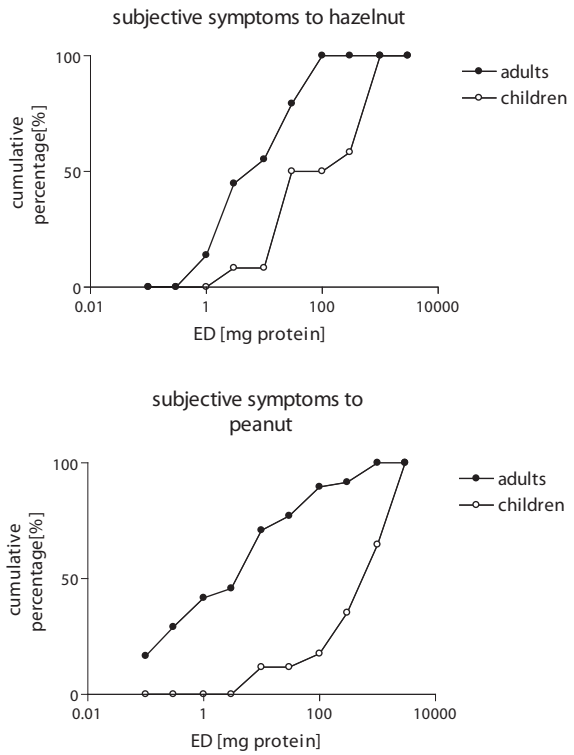
Taken together, a diagnosis based on sensitization and history, which in children is often deficient, would have overestimated the allergic population. Therefore, DBPCFC remains an important tool in the correct diagnosis of peanut and hazelnut allergy.

What is the value of eliciting doses (ED) determined by DBPCFC?

The dose after which the child reports the first symptoms during DBPCFC, either subjective (mild oral symptoms and occasionally nausea) or objective (other symptoms related to the skin, and gastro-intestinal, respiratory and circulatory system), is defined as the eliciting dose (ED). To allow determination of the ED, DBPCFC should start with a dose that is not likely to elicit any symptoms, so-called low dose DBPCFC. [19] We reported the first study with ED for hazelnut and peanut in children using low dose DBPCFC. ED in children have been investigated before, but reactions were already reported after the first administered dose, rendering accurate determination of ED impossible. [20-22] Our study showed that the individual ED for hazelnut and peanut varied widely, ranging from 5 mg to more than 1.5 g of peanut protein and from 1.5 mg to more than 500 mg for hazelnut protein. The lowest dose reported was 1.3 mg of peanut protein, comparable to our results. [20]

Usually, ED for subjective and objective symptoms are reported separately. [23-26] The ED for subjective reactions in our study were significantly lower than for objective symptoms; up to a 100-fold difference between the individual ED for subjective and objective symptoms was demonstrated. The ED for subjective reactions in our study were on average higher than in previous reports for hazelnut and peanut in allergic adults, although the lowest ED was comparable for hazelnut (1.5 versus 1 mg; Figure 1). [25] This does not necessarily mean that children are less sensitive to hazelnut and peanut than adults. Other factors may result in lower ED. The most likely factor is that adults

Figure 10.1



Eliciting doses for subjective symptoms in adults (●) and children (○) for A) hazelnut and B) peanut.

are more aware of subjective (oral) symptoms and report such symptoms earlier than children. In adult studies, a visual analog scale was used to determine the severity of oral symptoms after each portion, whereas in our study in children oral symptoms were reported by the child spontaneously. The eliciting doses for objective symptoms were on average also higher in children compared to adults. However, only 2 hazelnut-allergic adults (7%),[25] and 12 peanut-allergic adults (21%) from 2 studies developed objective symptoms,[24;26] in contrast to 67% of hazelnut-allergic and 91% of peanut-allergic children. This difference in frequency can be explained by the fact that DBPCFC in children were continued until objective reactions occurred, whereas DBPCFC in adults were stopped upon clear subjective symptoms on 3 subsequent doses. The lowest ED for objective symptoms to peanut was 10 mg protein in adults and 50 mg in children. For hazelnut, the lowest ED was 1 mg in adults, compared to 50 mg in children. The objective symptoms at these low doses in adults consisted of lip swelling, which can be regarded as relatively mild compared to the vomiting

and even respiratory distress that we observed in the children. Other factors that may affect the ED, such as the vehicle in which hazelnut and peanut is offered to the patient [27] or the way the hazelnut and peanut are processed [28-30] were similar for DBPCFC procedures in both adults and children at our clinic and are thus not the explanation for the observed difference.

ED can be useful to determine changes in sensitivity of a patient group at sequential time points. To date, one report has shown that the individual ED tend to remain stable over a period of 1 or 2 years without intervention. [31] Several studies that investigated potential therapeutic regimens for food allergy have used ED as outcome parameter. [32-34] It has been hypothesized that an increased ED would diminish the risk of developing allergic reactions to hidden food allergens. Although ED obtained from a patient group may be helpful in determining changes in the sensitivity of the group, the value of ED for an individual patient should be considered with sufficient caution. One report showed that ED and symptoms determined in a research setting (DBPCFC) do not resemble the severity of allergic reactions in daily life. [35] During exposure in daily life, differences in factors related to the patient, like physical condition, asthma status, concomitant medication and factors related to the food, such as the processing and vehicle, may account for this observation.

For that reason, children with hazelnut or peanut allergy should strictly avoid hazelnut and/or peanut in order to prevent unwanted accidental reactions in daily life. However, the difficulties with elimination of hazelnut and peanut are the hidden amounts due to contamination or inadequate labeling of food products. [36;37] Parents depend on labels describing the contents of the food product. If an allergen is not an ingredient the food product may still contain this allergen due to contamination during the production process. The amount of allergen present in such a food product can vary over a broad range. [38] As long as the debate about ''safe'' allergen thresholds in processed food products is not resolved, food manufacturers are forced to use 'may contain traces' on labels. 'May contain' labeling is defensive and may unnecessarily limit the choice of food products for patients. Meanwhile, consumers are increasingly ignoring this advisory labeling. [38] Knowledge of ED, food consumption patterns, the amount of contaminating allergen in food products [39-41] and the chance that an allergen is present in food products can be used to determine the risk of allergic reactions and to set limits for declaration of allergens for the food industry and regulatory authorities. [42]

Summarizing, ED can be determined by low dose DBPCFC. Information on ED in a group can be helpful in evaluating therapy, and improving food labeling strategies.

Determination of IgE binding to major allergens in hazelnut can predict the clinical reactivity to hazelnut

We showed that nearly half of the children with hazelnut sensitization developed allergic symptoms, of which one third had only mild oral symptoms. Children with only mild oral symptoms to hazelnut or without hazelnut allergy were consistently sensitized to Cor a 1, the Bet v 1 homologue in hazelnut, and/or Cor a 2, a profilin. In this group of children no sensitization to other hazelnut allergens was detected. Sensitization to Cor a 1 and Cor a 2 was associated with birch pollen sensitization, as can be expected in a birch endemic area. Cross-reactivity of Bet v 1 and Bet v 2 in birch pollen and Cor a 1 and Cor a 2 in hazelnut has been described previously, both in children and in adults from areas with birch trees. [43-46] Bet v 1 homologues are relatively heat unstable and not resistant to digestion. [47] They degrade in the oral cavity, which restricts symptoms in most cases to the oral mucosa. [45] Profilin sensitization seems not to be clinically relevant, although mild oral symptoms may also be related to this protein. [48] The fact that we observed sensitization to hazelnut profilin, Cor a 2, in association with mild oral symptoms or even absence of symptoms to hazelnut is in line with these reports.

In contrast, about two thirds of the hazelnut-allergic children developed more severe objective symptoms, such as urticaria and vomiting, suggesting the involvement of other hazelnut allergens than Cor a 1 and Cor a 2. In adolescents from Spain, sensitization to lipid transfer protein (LTP) in hazelnut, Cor a 8, was suggested to play a role in these more serious reactions. [49] In our group, sensitization to Cor a 8 was indeed restricted to children with objective reactions to hazelnut. LTP is a relatively small molecule of 8 kDa compared to other allergens in hazelnut. The compact structure may contribute to the resistance to digestion and heat, thereby conserving the allergenicity of this protein. [50;51] Sensitization to LTPs in other foods, such as peach, apple and grape has also been shown to be related to more serious allergic reactions. [52-54]

Until now, sensitization to LTP was only reported for the Mediterranean population, where birch trees are absent. In areas with birch pollen, sensitization to hazelnut (and apple) in adults is usually the result of primary sensitization to birch pollen. [45;52] In children from birch-endemic areas, sensitization to birch pollen often occurs from the age of 3, although sensitization to pollen can be observed at younger ages as well. [55-57] We therefore investigated our total population with sensitization to hazelnut from 0 to 17 years. The observation that a significant subset (34%) of these hazelnut-sensitized children was sensitized to LTP, and in particular young children not always in combination with sensitization to Bet v 1, suggests that these children became sensitized to

hazelnut before sensitization to birch pollen. Sensitization by hazelnut itself is unlikely, since it is not frequently eaten by children of that young age. Still, exposure to hazelnut in the environment, especially to hazelnut allergens that are stable and maintain their allergenicity, can not be excluded. Cross-reactivity to homologues of LTP in other foods might be an explanation, but IgE to LTP in peach was not detectable and the majority of children ate peach without problems. Moreover, all children tolerated apple, which also contains LTP. How these children became sensitized to hazelnut remains an interesting question.

The findings in our study indicate that sensitization to Cor a 8, an LTP, is associated with more severe objective reactions to hazelnut. In contrast, sensitization to Cor a 1 and Cor a 2 without additional sensitization to other hazelnut allergens is associated with mild oral symptoms or no symptoms at all. Determination of sensitization to individual hazelnut allergens instead of hazelnut extract may therefore predict the severity of hazelnut allergy and replace DBPCFC in the future.

Can determination of IgE binding to major allergens or peptides in peanut predict the clinical reactivity to peanut?

At least 81% of the peanut-sensitized children developed an allergic reaction during DBPCFC. Nine percent had only subjective symptoms and 91% developed objective symptoms. There was no difference in allergen recognition between these two groups. All children were sensitized to Ara h 2 and the majority to Ara h 6 as well. Other studies have confirmed the importance of sensitization to Ara h 2 and Ara h 6 in peanut-allergic subjects, although sensitization to Ara h 6 was not previously investigated in children. [26;58-60] Using SPT with purified allergens, Ara h 6 induced the largest reaction, also emphasizing that Ara h 6 should be regarded as a major peanut allergen in children as well. Both Ara h 2 and Ara h 6, being 2S albumins, are characterized by their stability, which explains their allergenicity. [61] In order to determine whether sensitization to major allergens could discriminate between children with mild or more severe reactions, sensitization to major peanut allergens was compared to the severity of the allergic reactions induced during DBPCFC. No specific major allergen was associated with mild or more severe reactions. Still, sensitization to multiple peanut allergens, including Ara h 1 and Ara h 3 was observed particularly in children with extensive clinical sensitivity to peanut.

With peptide micro-array immunoassays (MIA) it is possible to screen for the location of sequential IgE epitopes within these major peanut allergens. In our study, IgE epitopes were mainly located in Ara h 1 and Ara h 2, and to a lesser extent in Ara h 3. Each child showed a unique 'fingerprint'

of IgE binding. Specific single epitopes were recognized by the IgE of no more than 30-40% of our allergic subjects. The overall immunodominant regions were similar to previously reported IgE epitopes in each major allergen. [62-66] Neither did the distribution of the epitopes over Ara h 1, Ara h 2 and Ara h 3 in our data differ significantly from that reported previously. [67] Although it appeared that no immunodominant regions were related to more serious reactions, we did find an association between the severity of allergic reactions and the diversity of the IgE response towards Ara h 1, Ara h 2, and also Ara h 3. This finding is similar to previous reports describing an association between the number of allergens or epitopes recognized by IgE and the severity of allergic reaction to peanut. [26;67;68] The presentation of clustered multiple IgE epitopes to mast cells and basophils may result in a more efficient release of mediators. [69] Serum containing IgE to an increasing number of IgE epitopes in peanut allergens has been shown to lower the threshold for basophil and mast cell degranulation, as well as the extent of degranulation. [67] Hence, the diversity of IgE epitopes may relate to a more serious clinical outcome, as we observed in our study.

In summary, investigation of the response to major peanut allergens revealed that almost all peanut-allergic subjects were sensitized to purified Ara h 2 and Ara h 6. Using additional micro-array immunoassay (MIA) with sequential peptides derived from recombinant major peanut allergens, it was shown that IgE epitopes were mainly located on Ara h 2, but also on Ara h 1. Unfortunately Ara h 6 could not be tested, but our immunoblot and SPT data indicate that this allergen is also relevant. Children with more serious reactions displayed a more diverse polyclonal IgE response to peanut allergens and peptides, including Ara h 3. No specific allergens or epitopes were associated with the presence or absence of peanut allergy, therefore DBPCFC will remain necessary to diagnose clinical allergy.

Comparison of hazelnut and peanut allergy in children

Children with sensitization to hazelnut turned out to be less often allergic (46%) than children with peanut sensitization (81%). Moreover, hazelnut-allergic reactions appeared to involve considerably fewer objective symptoms (67%) than the allergic reactions to peanut (91%) during DBPCFC.

The observation that sensitization to hazelnut was not clinically relevant in a greater subset of children than sensitization to peanut is possibly explained by a selection bias toward the peanut-sensitized population. A history of a previous allergic reaction was more often reported by peanut-sensitized children than by hazelnut-sensitized children. However, the selection procedure for both was similar, and the frequency of previous allergic reactions to peanut in our study group was not

different from the frequency in the peanut-sensitized population in general from our clinic. This makes selection bias in the study groups less likely. However, a selection bias in favor of peanut allergy in our population (a tertiary clinic) compared to the general peanut-sensitized population in the Netherlands cannot be excluded.

An explanation for the observed difference between the severity of hazelnut and peanut-allergic reactions may be based on a difference in the frequency of cross-sensitization to birch or grass pollen. One third of the hazelnut-allergic population reported mild oral symptoms, and this was consistently associated with pollen cross-sensitization. This contrasts with the observation in the peanut group, in which only one out of 22 children was sensitized to grass and birch pollen and reported mild oral symptoms. Such mild oral symptoms related to pollen sensitization have only once been reported for peanut, in contrast to numerous reports for hazelnut. [70] Although sensitization to peanut allergens cross-reactive with birch and grass pollen, Ara h 8 (Bet v 1 homologue) and Ara h 5 (profilin), [70;71] was not investigated, no IgE binding was detected on immunoblots to proteins that may represent these cross-reactive allergens. Apparently, such cross-sensitization with pollen seems to occur less in our peanut-sensitized pediatric population than in our hazelnut-sensitized population. This observation may not only be an explanation for the difference in severity of hazelnut-allergic reactions, but also for the difference in clinical relevance (46% for hazelnut vs. 81% for peanut). It has been demonstrated that sensitization to hazelnut with concomitant sensitization to birch pollen is not necessarily clinically relevant. [72;73]

A subset of children with hazelnut sensitization developed objective symptoms. These objective symptoms and the ED were similar to symptoms elicited by peanut. Remarkably, the hazelnut-allergic children with objective symptoms were all sensitized to peanut, and also to multiple other nuts (Brazil nut, walnut, pecan, almond, cashew, pistachio). This observation is in accordance with other studies in children describing that in young children sensitization to hazelnut coincides with sensitization to peanut. [4;74;75] Children with objective reactions to hazelnut were not only sensitized to Cor a 8, but also to numerous other allergens in hazelnut as shown on the immunoblots. These may account for the co-sensitization observed among peanut and hazelnut. In a recent study, linear surface-exposed IgE-binding epitopes identified in Ara h 3 were shown to exhibit structural homology with Cor a 9 in hazelnut, but also with other allergenic tree nuts (Jug r 4 of walnut and Ana o 2 of cashew nut). [76] In line with this finding, sensitization to Ara h 3 in our study was strongly associated with sensitization to hazelnut, and especially with non-pollen related hazelnut sensitization. The precise role of Ara h 3 and other allergens possibly involved in the observed co-sensitization among hazelnut and peanut has yet to be defined.

In conclusion, the different background of hazelnut sensitization, showing marked cross-reactivity with pollen, as compared to peanut sensitization, may account for the difference in clinical relevance of sensitization between hazelnut and peanut.

T cell responses to peanut in relation to specific IgE and IgG4: what is the impact on future therapeutic options?

Children with peanut allergy display a maladaptive immune response characterized by IgE production to major peanut allergens. We demonstrated that the T cell response to these major allergens in peanut-allergic subjects was characterized by a Th2 response, as was expected for allergy. Peanut-sensitized subjects without peanut allergy also displayed a Th2 response to major peanut allergens, although to a lesser extent than peanut-allergic subjects. This was in line with the finding that these children showed IgE binding to peanut as well. The fact that sensitized children without allergy showed a Th2 response has been reported previously for peanut. [77]

Remarkably, despite the dominant IgE response to purified Ara h 2, the T cell response to Ara h 2 was less enhanced than to the major peanut allergens Ara h 1, Ara h 3 and Ara h 6. This is particularly striking when taking into account the 70% homology between Ara h 2 and Ara h 6, [59] suggesting that Ara h 6 contains T cell epitopes in a region that is not homologous with Ara h 2. A possible explanation for the low response to Ara h 2 can be that the simultaneous exposure of multiple peanut allergens during exposure to peanut may provide bystander help for the IgE response to Ara h2. The glycan structures on Ara h1 for instance can act as a ligand for dendritic cells, thereby creating a Th2-skewing environment which may skew the T cell response towards other peanut allergens as well. [78]

In order to investigate possible therapies that could divert the peanut-specific response, probiotics were used as a non-specific modulator of the immune system. [79] Although the *in vitro* results showed Th1 and regulatory responses to peanut in allergic subjects, the clinical outcome after *in vivo* administration was disappointing. No increase in Th1 or regulatory cytokines upon stimulation with peanut was observed in the treatment group. The only significant clinical effect of probiotics that has been demonstrated in several studies with respect to allergic disease is a possible prevention of eczema. [80-82] Whether an immunological effect of probiotics is responsible for the observed preventive effect remains uncertain. In our study, probiotics did not appear to be able to divert an already existing allergen-specific response.

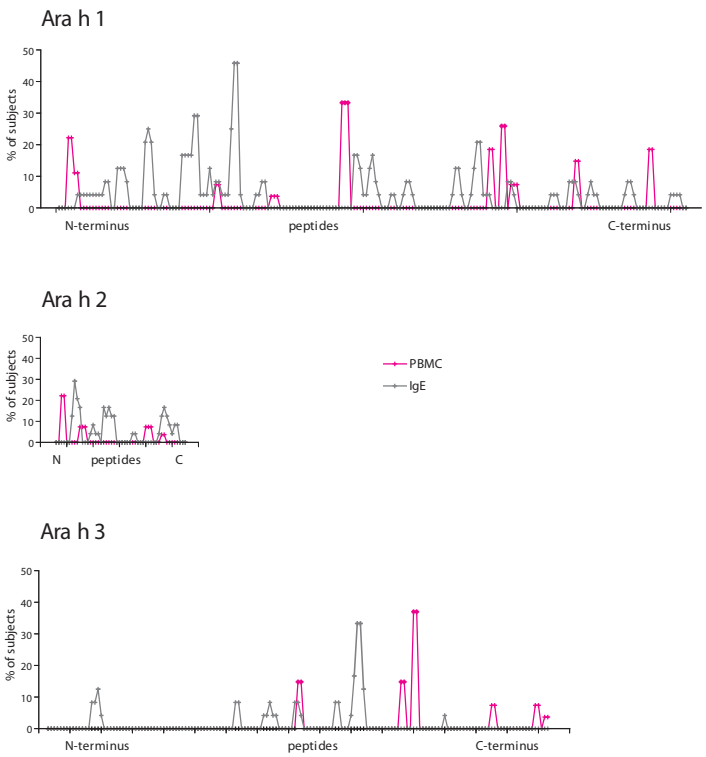
The only available therapy for IgE-mediated hypersensitivity at this moment is allergen-specific

immunotherapy, currently used for inhalant and insect venom allergies. [83] During immunotherapy tolerance can be induced, and is associated with induction of allergen-specific IL-10 and IgG4. [84;85] It has been suggested that allergen-specific IgG4 competes with IgE binding, thereby modulating the adaptive immunity towards the allergen. [86] In our study, both peanut-allergic and non-allergic peanut-sensitized children had remarkably low levels of IgG4, together with low levels of IL-10. These low levels of IgG4 may be a reflection of very low exposure to peanut during elimination diets prior to DBPCFC. In the contexts where IgG4 has been correlated with protection, such as in cat allergy, IgG4 is associated with chronic antigen exposure. [87] Ongoing exposure to peanut during immunotherapy has been shown to increase IgG production in peanut-allergic subjects. [88] However, these attempts to develop immunotherapy for peanut had to be halted due to an unacceptably high rate of (serious) allergic reactions. This suggests that an immunotherapeutic extract should preferably contain relevant allergen or allergen fragments that leave the T cell response intact while preventing IgE binding. It was shown previously that by substituting one amino-acid in relevant IgE epitopes in major peanut allergens, IgE binding to that epitope was inhibited, whereas the T cell response was maintained. [62;63;66] In order to completely prevent IgE binding to peanut, numerous IgE epitopes should be modulated because the IgE epitope 'fingerprint' is unique for each individual and is spread throughout Ara h 1-3. Ara h 6 was not investigated by MIA, but it is likely that numerous IgE epitopes are located on Ara h 6 as well. Although it will be difficult to modulate major peanut allergens in such a way that IgE binding is inhibited for all peanut-allergic subjects, promising results with this approach were demonstrated in murine models. [89] Whether the IgE binding in these mice was as diverse as in peanut-allergic patients has not been described. The development of modified immunotherapy might be facilitated if it were possible to use only one allergen. Based on our data, Ara h 2 seems to be the most relevant peanut allergen, because this allergen was recognized by all peanut-allergic children. However, Ara h 2 was unable to induce a significant T cell response by itself, suggesting that other peanut allergens should be used in combination with Ara h 2.

Another option is the use of several potent peptides that can induce T cell responses, as have been used for the development of vaccines and immunotherapy for bee and cat. [90-92] A computer model was used to predict pan-DR binding epitopes in peanut allergens. [93] We showed that several of the peptides selected in this way were able to induce T cell responses. The T cell responses of allergic subjects towards peptides in our study were less pronounced than those of control subjects. The frequency of peptide-specific T cells in these allergic children was too low to be detectable in the circulation, most likely due to prolonged elimination of peanut. Other studies have shown

that T cell responses to peptides do not differ between allergic subjects and healthy controls. [94-96] Moreover, the HLA types in children with peanut allergy are comparable to the general population. [97] Therefore, differences with regard to the specific peptides being recognized as compared to healthy subjects are not expected. A limitation of the pan-DR prediction model is that peptides that preferentially bind to other HLA molecules are not taken into account. One study reported that presentation of certain cow's milk peptides was restricted to HLA-DQ, indicating that other peptides might be relevant as well. [98]

Figure 10.2



Sequential peptides of 20 aa with an offset of 3 aa, derived from A) Ara h 1, B) Ara h 2 and C) Ara h 3, are displayed. IgE epitopes for allergic subjects are indicated with a grey line. The orange line represents peptides that induced a T cell response in either allergic or healthy subjects.

An interesting observation in our study was that peptides derived from Ara h 1, Ara h 2, Ara h 3 and Ara h 6 that did induce a T cell response only in part overlapped with IgE binding regions as shown by MIA (Figure 2). When considering peptide immunotherapy, a partial overlap will reduce the risk of IgE binding. This, together with the fact that such short peptides are unable to crosslink IgE, could contribute to the safety of this approach.

In conclusion, children with peanut allergy display a Th2 response to peanut allergens. Non-specific stimulation with probiotics was insufficient to modulate this response towards Th1. Inducing tolerance by peanut-specific immunotherapy remains a more promising option. Preferably, multiple major peanut allergens should be used. Modification of these allergens seems difficult due to the wide variation of IgE reactivity between individuals, but peptide immunotherapy seems an interesting option, for which further research is indicated.

Concluding remarks

Determination of sensitization to hazelnut or peanut alone is not sensitive enough to establish a correct diagnosis of food allergy in children. Taking into account the lifelong prognosis and associated impact on daily life of this disease, a correct diagnosis is essential. Determination of specific IgE to several major allergens/components in hazelnut revealed that children with only detectable IgE binding to cross-reactive allergens with pollen did not develop objective symptoms. For this particular population this method may be used as component resolved diagnosis, thereby reducing the need for DBPCFC in the future. In contrast to hazelnut, neither IgE to specific major peanut allergens, nor IgE to specific epitopes was associated with the outcome of DBPCFC. We demonstrated that IgE polyclonal diversity was more pronounced in children with more serious reactions, but this is insufficient to replace DBPCFC. The allergic subset of peanut-sensitized children in our study was relatively large. Perhaps by extending the non-allergic peanut-sensitized population, the value of sensitization to major peanut allergens in discriminating between absence and presence of peanut allergy may become more obvious. For now, DBPCFC remains an important tool in the correct diagnosis of peanut allergy.

A history of previous allergic reactions to hazelnut or peanut might improve the sensitivity of diagnostic tests. However, many children were not aware of previous exposure to hazelnut or peanut. An interesting question remains to how these children became sensitized. Hazelnut sensitization that was associated with objective reactions may be related to peanut instead of pollen, with a possible role for cross-reactivity between Ara h 3 and Cor a 9. Determination of IgE reactivity to hazelnut and peanut allergens and peptides at sequential time points in a prospective birth cohort may reveal when and to which allergens these children become sensitized. This information can be useful for a better understanding of the development of hazelnut and peanut allergy in young children and for the timing of prevention measures or treatment in the future.

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7

Summary & samenvatting

Summary

The word allergy is used colloquially to indicate any undesirable reaction to foods or other proteins that enter the body from the outside, also called allergens. In this thesis the term allergy is used to denote reactions that are mediated by the antibody IgE. Such allergic reactions usually take place within half an hour after ingestion and may include itching of the mouth, swelling of the throat, hives, swelling of the face, stomach ache, vomiting, diarrhea, hay fever symptoms, obstructed breathing, and shock. Fortunately, it is rare for allergic reactions to foods to be fatal. In patients who develop allergic symptoms after eating certain foods, IgE specific for the offending food can be shown. The presence of IgE antibodies against these allergens is called sensitization. Sensitization can be demonstrated with a blood or skin test.

Sensitization to hazelnut and peanut is common during childhood, especially in children with eczema. Although hazelnut and peanut belong to different families of plants, concurrent sensitization to both is not unusual. Skin tests and the level of specific IgE in the blood, methods to measure sensitization, have low specificity. That is, people who do not have symptoms after eating hazelnut or peanut may still test positive, indicating that they are sensitized. Striking is the fact that young children who have never eaten hazelnut or peanut still may have become sensitized. The clinical relevance of sensitization without dietary exposure is unclear. A challenge, preferably a double-blind placebo-controlled food challenge (DBPCFC) with the relevant food is then indicated, as this is the only way to diagnose food allergy with certainty. This prevents children who are sensitized but not clinically allergic being put on a restrictive diet, often for many years.

Hazelnut and peanut allergy have a negative influence on quality of life; therefore a correct diagnosis is mandatory. In chapter 2 we investigated whether the impact of food allergy on daily life has to do with the constant danger of an allergic reaction and the fear that a child might even die. By means of questionnaires given to parents before and after challenge, we studied this fear. It appeared that parents indeed experienced a high degree of anxiety, comparable to that of a pregnant mother who lost a child to crib death. It is striking that after the challenge, regardless of the results, parents were less anxious.

The importance of challenges in diagnosing hazelnut allergy is discussed in chapter 3. More than half of the children who were sensitized to hazelnut turned out not to be hazelnut-allergic and no longer were obligated to follow a hazelnut-avoidance diet. One third of the children who did have symptoms, and thus were hazelnut-allergic, had only mild complaints, including itching and swelling of the mouth. The other two thirds developed more serious symptoms, such as generalized

hives and vomiting. In addition to hazelnut this last group was also sensitized to other nuts, such as walnut, pecan, almond, Brazil nuts, pistachio nuts, cashew and also to peanut.

To refine the diagnosis of hazelnut allergy, in chapter 4 sensitization to the specific allergens in hazelnut was studied in this group of children. Some of the children who were asymptomatic and all of those who had only mild symptoms were sensitized to the unstable allergens Cor a 1 and Cor a 2, which manifest a high degree of homology to the allergens in birch pollen, Bet v 1 and Bet v 2. All of the children with more serious complaints turned out, in addition to Cor a 1 and Cor a 2, to be sensitized to a very stable allergen called "lipid transfer protein" (LTP, or Cor a 8). The sensitization pattern for hazelnut allergens seems predictive of the results of a challenge, and in future may even replace it.

The clinical relevance of sensitization to peanut is discussed in chapter 5. By performing challenges, peanut allergy was demonstrated in about 80% of the children who were sensitized to peanut. Sensitization to peanut therefore appears to be more often clinically significant than sensitization to hazelnut. Symptoms during challenge ranged from mild oral complaints to severely obstructed breathing (13%) and falling blood pressure (5%). We also studied the threshold values at which the first symptoms appeared. These threshold values varied widely from ca. 10 mg of peanut flour to 10 grams of whole peanuts. The lower the threshold value the greater the risk of a reaction to hidden traces of peanut in processed foods.

We further refined the diagnosis of peanut allergy by investigating the sensitization to individual peanut allergens. In chapter 6 we show that allergic children primarily recognized the stable 2S albumins in peanut, Ara h 2 and Ara h 6, and to a lesser degree, Ara h 1 and Ara h 3. However, we did not distinguish any differentiation in symptomology on the basis of patients' pattern of sensitization to specific allergens, as was the case with hazelnut allergy. The pattern of sensitization for each individual child remained stable over a period of 20 months after challenge.

In chapter 7 we investigated whether the severity of a reaction to peanut was related to the sensitization to specific IgE-binding epitopes (an epitope is a recognition site on an allergen that can be specifically recognized by antibodies) on the allergens Ara h 1, Ara h 2 and Ara h 3. This was not the case. The severity of an allergic reaction did appear to be correlated with the number of epitopes that were recognized. This pattern of IgE binding to epitopes also remained stable over time.

Subsequently in chapter 8 we studied the activation of T cells by peanut allergens and possible T cell epitopes. Notable was that the response to Ara h 2 was relatively low, in contrast to the strong response to the other peanut allergens, Ara h 1, Ara h 3 and Ara h 6. This response was characterized

by proliferation of T cells and an elevated production of IL-13, a cytokine that stimulates B cells to produce IgE. The epitopes that induced a response were only partially overlapping IgE-binding epitopes. This is an important discovery when it comes to developing peanut-specific immunotherapy. Stimulating T-cell reactivity is necessary for immunotherapy to be successful. However, when using intact peanut allergens for immunotherapy, IgE-mediated reactions often occurr, making this form of immunotherapy too dangerous at present. If the B cell response (IgE reactivity) can be reduced by using a selection of peptides, the side effects of such treatment may also be reduced to a considerable extent. This may allow the application of immunotherapy for food allergy in the future.

In chapter 9, we investigated the effect of probiotics on the T cell reactivity of children with multiple food allergies including peanut allergy. Probiotics, including lactic acid bacteria that naturally occur in the bowel, are said to be beneficial to the immune system. Adding probiotics to the diets of these children however failed to change their T cell reactivity or degree of sensitization to peanut. Thus probiotics do not appear to offer an option for the treatment of peanut allergy.

This thesis shows that oral challenges remain an important tool in ascertaining hazelnut and peanut allergy. In addition, it demonstrates that in hazelnut allergy the specific allergen that is recognized determines the clinical relevance of sensitization, while for peanut allergy it is the number of allergens recognized that seems to be paramount. The fact that the B cell reactivity can be reduced by the use of a selection of non-IgE binding peptides, whereas T cell reactivity can be preserved, is promising toward the development of safe allergen-specific vaccines to be used in immunotherapy.

Samenvatting

Het woord allergie wordt in de volksmond gebruikt voor elke ongewilde reactie op voedsel of andere eiwitten uit onze omgeving, ook wel allergenen genoemd. In dit proefschrift wordt de term allergie gebruikt voor reacties die gemedieerd worden door de antistof IgE. Dergelijke allergische reacties ontstaan meestal binnen een half uur na inname van het bewuste voedingsmiddel en omvatten jeuk in de mond, zwelling van de keel, jeukende galbulten, zwelling van het gezicht, buikpijn, braken, diarree, hooikoortsachtige klachten, benauwdheid en shock. Gelukkig overlijden er slechts zeer zelden mensen ten gevolge van een voedselallergie. Bij personen met allergische symptomen na het nuttigen van bepaald voedsel kan IgE worden aangetoond dat specifiek is voor allergenen in het betreffende voedingsmiddel. De aanwezigheid van IgE antistoffen gericht tegen deze allergenen wordt sensibilisatie genoemd. Sensibilisatie kan worden aangetoond door middel een bloedtest of een huidtest.

Sensibilisatie voor hazelnoot en pinda komt vaak voor op de kinderleeftijd, vooral bij kinderen met eczeem. Hoewel hazelnoot en pinda tot verschillende plantenfamilies behoren, is gelijktijdige sensibilisatie voor beide niet zeldzaam. De betekenis van een sensibilisatie is vaak onduidelijk. De specificiteit van de bepaling van specifiek IgE in het bloed en een huidtest is namelijk laag. Dat wil zeggen dat ook personen zonder symptomen na het eten van hazelnoot of pinda een positieve test kunnen hebben, oftewel gesensibiliseerd kunnen zijn. Opvallend is dat jonge kinderen die nog geen pinda's of hazelnoot hebben gegeten toch al gesensibiliseerd zijn. Bij sensibilisatie zonder duidelijke blootstelling is het onduidelijk wat de klinische relevantie van sensibilisatie is. Een provocatie, bij voorkeur een dubbelblinde placebo-gecontroleerde voedsel provocatie met het betreffende voedingsmiddel is dan aangewezen, omdat dit de enige manier is om een juiste diagnose te stellen. Dit voorkomt dat kinderen met een klinisch irrelevante sensibilisatie, meestal voor een langere tijd, een dieet vrij van pinda en noten krijgen. Hazelnoot- en pinda-allergie hebben een negatieve invloed op de kwaliteit van leven, ook daarom is een juiste diagnose onontbeerlijk. In *hoofdstuk 2* werd onderzocht of de impact van voedselallergie op het dagelijks leven te maken heeft met het constant aanwezige risico op een allergische reactie en de angst dat een kind daar zelfs aan kan overlijden. Door middel van vragenlijsten aan ouders werd vóór en na provocatie deze angst bestudeerd. Het bleek dat ouders inderdaad een hoge mate van angst ervaren, vergelijkbaar met zwangere moeders die eerder een kind verloren hebben aan wiegendood. Opvallend was dat na provocatie, ongeacht de uitkomst ervan, ouders minder angst voor blootstelling aan hazelnoot en pinda hadden.

Het belang van provocaties bij de diagnostiek van hazelnootallergie wordt in *hoofdstuk 3* besproken. Meer dan de helft van kinderen met sensibilisatie voor hazelnoot bleek geen klachten te ontwikkelen en hoefde daarom voortaan geen dieet meer te volgen. Van de kinderen die wel allergische symptomen hadden, en dus hazelnootallergie, kreeg éénderde alleen milde klachten van jeuk en een gevoel van zwelling in de mond. Tweederde ontwikkelde daarnaast meer ernstige klachten, zoals galbulten over het hele lichaam en braken. Deze laatste groep bleek naast een sensibilisatie voor hazelnoot ook sensibilisatie voor andere noten te hebben, zoals walnoot, pecan, amandel, paranoot, pistache, cashew en ook voor pinda.

Om de diagnostiek voor hazelnootallergie te verfijnen werd in *hoofdstuk 4* de sensibilisatie voor verschillende allergenen in hazelnoot bestudeerd in bovengenoemde kinderen. Kinderen met alleen milde klachten, en ook een deel van de kinderen zonder klachten waren gesensibiliseerd voor de labiele allergenen Cor a 1 en Cor a 2 die grote gelijkenis vertonen met allergenen in berkenpollen: Bet v 1 en Bet v 2. Het bleek dat alle kinderen met ernstige klachten naast Cor a 1 en Cor a 2 ook gesensibiliseerd waren voor een zeer stabiel klein allergeen genaamd "lipid transfer protein" (LTP of Cor a 8). Het patroon van sensibilisatie voor allergenen in hazelnoot lijkt de uitkomst van een provocatie voorspellen, en zou deze mogelijk in de toekomst zelfs kunnen vervangen.

De relevantie van sensibilisatie voor pinda wordt besproken in *hoofdstuk 5*. Bij ongeveer 80% van de kinderen met sensibilisatie voor pinda werd met behulp van provocatie een pinda-allergie aangetoond. Daarmee lijkt sensibilisatie voor pinda vaker klinisch relevant dan hazelnoot sensibilisatie. De klachten tijdens provocatie varieerden van milde klachten in de mond tot forse benauwdheid (13%) en daling van de bloeddruk (5%). De drempelwaarde waarop de eerste klachten optraden werd eveneens bestudeerd. Deze drempelwaarde vertoonde grote individuele variatie, van ca 10 mg pindameel tot 10 gram hele pinda's. Hoe lager de drempelwaarde des te groter de kans is om een reactie te ontwikkelen op verborgen sporen van pinda in samengestelde voedingsmiddelen.

De diagnostiek voor pinda-allergie werd eveneens verfijnd door sensibilisatie voor afzonderlijke pinda-allergenen te onderzoeken. *Hoofdstuk 6* laat zien dat allergische kinderen voornamelijk de stabiele 2S albumines in pinda, Ara h 2 en Ara h 6, herkennen en in mindere mate Ara h 1 en Ara h 3. Echter, het patroon van sensibilisatie voor specifieke allergenen maakte geen onderscheid in klachten zoals dat voor hazelnoot werd gezien. Het patroon van sensibilisatie per kind bleef stabiel gedurende een periode van 20 maanden na provocatie.

In *hoofdstuk 7* werd onderzocht of de ernst van de klachten voor pinda was gerelateerd aan sensibilisatie voor specifieke IgE-bindende epitopen (een epitoop is een herkenningsplaats op het

allergeen dat specifiek kan worden herkend door antistoffen) van de allergenen Ara h 1, Ara h 2 en Ara h 3. Dit was niet het geval. Wel bleek de ernst van de allergische reactie gecorreleerd te zijn met het aantal epitopen dat herkend werd. Dit patroon van IgE binding aan epitopen bleef stabiel in de tijd.

Vervolgens werd in *hoofdstuk 8* de activatie van T cellen door pinda-allergenen en mogelijke T cel epitopen bestudeerd. Opvallend was dat de respons op Ara h 2 betrekkelijk laag was, in tegenstelling tot de sterke respons op de andere pinda-allergenen, Ara h 1, Ara h 3 en Ara h 6. Deze respons werd gekenmerkt door proliferatie van T cellen en een verhoogde productie van IL-13, een cytokine dat B cellen aanzet tot de productie van IgE. De epitopen die een respons induceerden waren slechts voor een deel vergelijkbaar met IgE bindende epitopen. Dit is een belangrijk gegeven met het oog op de ontwikkeling van pinda-specifieke immunotherapie. Het stimuleren van T cel reactiviteit is een voorwaarde voor succesvolle immunotherapie. Bij het gebruik van intacte pinda-allergenen voor immunotherapie treden echter vaak IgE-gemedieerde reacties op, waardoor deze vorm van immunotherapie op dit moment met teveel risico's gepaard gaat. Wanneer door gebruik van een selectie van peptides de B cel respons (IgE reactiviteit) kan worden verminderd, zullen de bijwerkingen van een dergelijke behandeling ook aanzienlijk worden verminderd. Daardoor kan in de toekomst immunotherapie toch een optie voor de behandeling van voedselallergie zijn.

Aan probiotica, onder andere melkzuurbacteriën die van nature in de darm voorkomen, worden gunstige effecten op het immuunsysteem toegeschreven. Daarom werd in *hoofdstuk 9* onderzocht wat het effect van probiotica was op de T cel reactiviteit van kinderen met meerdere voedselallergieën, waaronder pinda-allergie. Het gebruik van probiotica als toevoeging aan de voeding van deze kinderen veranderde echter de T cel reactiviteit en mate van sensibilisatie op pinda niet. Probiotica lijken dus niet geschikt voor de behandeling van pinda-allergie.

Dit proefschrift laat zien dat orale provocaties een belangrijk middel zijn om hazelnoot- of pinda-allergie vast te stellen. Daarnaast toont dit proefschrift aan dat het specifieke allergeen in hazelnoot dat herkend wordt de klinische relevantie bepaalt, terwijl bij pinda-allergie het veel meer gaat om het aantal allergenen dat herkend wordt. Het gegeven dat de B cel reactiviteit kan worden verminderd door gebruik te maken van een selectie niet-IgE bindende peptiden, terwijl tegelijkertijd de T cel reactiviteit behouden blijft, geeft hoop op de ontwikkeling van veilige allergeen-specifieke immunotherapie.

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Acknowledgements

Introduction

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Mijn opa, een autodidactische zeiler, was van mening dat je door zorgvuldig observeren veel kunt leren. Door zijn belangstelling voor de geneeskunde en filosofische instelling was ik gemotiveerd om aan een promotietraject te beginnen. Opa, bedankt voor je aandacht en kritische noten in de lange tijd dat we elkaar hebben gekend.
Lieve pap en mam, bedankt voor de vrijheid en het vertrouwen dat jullie me hebben gegeven om zelf de koers te bepalen, waarheen dan ook. Mocht het even niet meteen lukken dan is het erg fijn om bij jullie te kunnen mopperen. Een lekker kopje thee, mooie film of uitwaaien op het water in Zeeland; kortom thuis met jullie doet wonderen.

Aan het eind van mijn co-schap dermatologie opperde Suzanne Pasmans dat ik onderzoek kon doen. Nu zijn we ineens zes jaar verder, verweerd door de zilte zeelucht met zowaar een gevuld logboek. Suzanne, ik heb respect voor het doorzettingsvermogen en het enthousiasme waarmee je aan projecten begint, soms tegen de wind in, met uiteindelijk hele mooie resultaten! Voor mij ben je de hele tocht een stuurvrouw niet alleen op wetenschappelijk maar zeker ook op persoonlijk vlak geweest.
In de zomer van 2001 had ik een aantal vragen over promoveren en dermatologie. In plaats van het secretariaat kreeg ik per ongeluk Professor Bruijnzeel aan de telefoon. Carla, ik kan me nog goed herinneren dat je welgeteld een halfuur de tijd voor me nam. Dit resulteerde kort daarop in een telefoontje terug met de vraag of ik bereid was onderzoek te komen doen. Dankzij jou bleef het vaste punt op de wal zichtbaar zodat we een rechte koers bleven varen, het zogenaamde focusseren. Onder jouw leiding zal ik me nu op de dermatologie gaan focussen. Maar eerst een toost op 1 november, een prachtige dag!
Bij de officiële kennismaking in de kamer van Carla werd ik voorgesteld aan Els van Hoffen, co-promotor. Samen maakten we een eerste uitstapje naar de wereld van de probiotica, maar bij de macrobiotische congressen zijn we afgehaakt. Vooral in de laatste 2 jaar, toen de focus van het onderzoek zich verplaatste van de kliniek naar het lab, was jij een waardevolle stuurvrouw. Helder

en rustig, met plaats voor humor, legde je de technische details uit. Els, je expertise, je Engels - zelfs beter dan sommige Amerikanen – en je zorgvuldigheid heeft van mijn kladjes een paar prachtige artikelen gemaakt.

Methods

Study population

Alle deelnemende kinderen, in totaal zeker 70, en hun ouders; petje af voor de durf en flexibiliteit om aan deze tocht te beginnen, én af te maken. Het was spannend maar gelukkig ook de moeite waard. Ontzettend bedankt.

Van alle patiëntjes is ruimschoots materiaal verzameld. Helaas was dit niet altijd voldoende. De serumbank, een behoorlijke klus voor Kees en Ans, was een uitkomst om de ietsepietsie restjes serum in de vriezer terug te vinden.

DBPCFC

"Heerlijk naïef" begon ik op afdeling Pauw onder begeleiding van Yolanda Meijer, kinderarts. Na honderd koemelk- en kippeneiprovocaties en een cursus Advanced Pediatric Life Support was ik dan eindelijk klaargestoomd voor de hazelnoot- en pindaprovocaties. Ondertussen had jij, Yolanda, de dankbare taak om de patiëntjes van dokter Wauters over te nemen. Mijn complimenten hoe je dat hebt gedaan. Helaas hebben jullie allebei de weddenschap verloren, maar ik blijf de kindergeneeskunde een warm hart toedragen. Proost op de samenwerking!

Met de intrede van Maarten Hoekstra in het WKZ ging het ineens voor de wind met de provocaties. Maarten, alle lof voor hoe jij als co-promotor op diplomatieke wijze de provocaties en huidpriktesten hebt ingepland en begeleid, met recht een kundig stuurman. Hoe druk ook, ik kon altijd op je rekenen bij een allergische reactie van een kind. Ook met het nakijken van alle manuscripten was je steevast de eerste die reageerde.

Alle testpersonen op poli blauw en de stafgang van de dermat; jullie ongezouten mening op de uitgebreide smaaktesten was waardevol. Een aantal kinderen was echter een andere mening toegedaan…Voor oplossingen voor moeilijke eters, en vragen over dieet en sporenexpositie was er diëtiste Ems. Hartelijk dank voor je adviezen.

Afdeling Pauw, gerund door een stel adequate dames en één heer. Jullie hebben al jullie vaardigheden uit de kast moeten halen om die (veel te) grote porties pap te voeren. Dat pak koffie voor de lekkerste capuccino's van het WKZ tijdens een welverdiende pauze zal ik binnenkort

eindelijk eens komen brengen.
Inmiddels heeft Petra Kentie het roer overgenomen. Petra, super dat de provocaties nog steeds plaatsvinden! Succes met de laatste resten van de enorme wachtlijst.

Questionnaires

Met Monique en Wieneke van de psychosociale afdeling is er tijdens brainstormsessies genoeg bedacht voor de komende 10 jaar. Nu alleen de financiering nog! Het is een verlies voor het WKZ dat jullie naar het Westen van het land vertrokken zijn. Wien, gelukkig hebben we het afgesloten met een tripje naar San Diego. Binnenkort maar weer eens brainstormen over hoofdstuk 2, en bijkletsen. Of andersom.

Skin prick tests

Dolly, José, Margreet en Judith van poli blauw, jullie hebben hemel en aarde bewogen om een deel van de huidpriktesten te organiseren. Zelfs zo goed dat tot mijn vreugde het handgeschreven logboek met huidpriktest-uitslagen is vervangen door een digitale versie!

Immunoblotting

Stansie, al bijna een ouwe rot in het vak, ik weet nog hoe je begon. Met rode konen zeer geconcentreerd de juiste sera in de juiste buisjes pipetteren: 1-2-3-6. Nadat we de felbegeerde grammen eiwit hadden bemachtigd, volgden blotjes en eindeloze T cel proeven. Alle data in dit boekje staan door jouw nauwgezetheid als een huis! Tijdens onze gezamenlijke tripjes waren we een stuk onhandiger met ontzettende Harriette acties…
In de zomer van 2005 hebben Stans en ik het ruime sop gekozen om warm onthaald te worden in het CLB bij Ronald, Serge en familie Jaap & Laurian met alle kids. Met de kwaliteiten van Jaap als verstekeling konden we onze eigen sera zelf labelen in de radio-actieve kamer. Volgens mij zijn we nu echt klaar met de hazelnoot blotjes Jaap! Of toch nog even Cor a 9…?
Eenmaal klaar met blotten komt het belangrijkste: het beoordelen. Stef is de expert op dit gebied, die mij eindelijk heeft uitgelegd waar die ‘S’ in 2S albumines nu precies voor staat.

Micro imuno-arrays

For this technique we collaborated with an overseas lab in New York. Wayne, thank you for the hospitality and expertise in ‘your’ lab, and for your problem solving capacities. After years of patience, we finally have our article (almost) accepted by JACI! Don’t forget to bring your clogs to the Netherlands.

Working in the lab at the 17th floor, although for a short period, was a great experience. Luda, thank you for your kind support. Andrea, you are one of the few people I know that can get excited about either micrograms of peanut or bargain handbags. Doerthe, the working environment has certainly improved; last time I checked no dangerous kettles were boiling water to pump up the humidity. I am looking forward to collaborating again with you in the future.

T cells

De eindeloze golfbeweging in proliferaties van T cellen, kielhalen die eigenwijze lymfocyten! Overigens Berent, Mark en Wilco van het immunolab in het WKZ, en Adrie, Els en Stans: bedankt voor alle adviezen en pogingen om de T cellen in het gareel te krijgen en houden.

Results

Bij het genereren van de eerste data kwam André Knulst steeds meer in zicht. Ervaren en precies op de hoogte van de laatste ontwikkelingen op het gebied van voedselallergie was jij als co-promotor de dieselmotor achter de klinische artikelen. Je hebt me soms tot het uiterste gedreven, waardoor ik het beste uit mezelf moest halen. Daarvoor heb je me de ruimte en tijd gegeven, wat ik waardeer en waarvoor ik je erg dankbaar ben!

Voor het bespreken van leuke resultaten aan de horizon is Edward de juiste persoon. Overigens, ook bij minder leuke data verlaat iedereen met een positief gevoel jouw kamer. Door je enthousiasme begin ik dan toch weer aan een schijnbaar goed verhaal. Ik vind het nog steeds geweldig dat het NYC project zo goed uit de verf is gekomen.

Kennis over voedselallergie is natuurlijk onontbeerlijk. Gelukkig waren er mede-onderzoekers in hetzelfde schuitje met de nodige ervaring. Vooral Kim, met nagenoeg hetzelfde project, was een ideaal voorbeeld. Uiteraard werd er in de voedselallergiekamer voornamelijk over voedselallergie gepraat, maar ook over de meest uiteenlopende andere zaken. Op nummer één staat toch wel de huiskooplust die werkelijk iedereen heeft besmet. Els, Stans, Bert, Titia, Kim, Maarten, Adrie, Jaap, zelfs daarbuiten ook Machteld, Suzanne en Marjolein: succes met alle verhuisplannen, verbouwingen en inburgeringscursussen in de nieuwe omgeving. Voor het echte denkwerk is de koptelefoon een uitkomst, met wat lekkere meezingers van Kim of de collectie op iTunes van buurman Maarten. Na werktijd gaat die weer af om beregezellig nog een biertje te drinken in de Basket of Primus. Helemaal leuk Master Bert!

Ook altijd van de partij bij de koffie of de borrel zijn het komisch duo Onno en Peter, met in hun

kielzog de dames Inge en Sara en kapitein overzees Dirk Jan van de eczeemkamer. Niet te vergeten Maurice, Marloes, Ans, Annemieke, Ines, Jantine, Miranda, Stefan, Igor en Ilse als bemanning van de stafgang. Jullie waren goed voor de nodige afleiding en ontspanning, zeker op sportief gebied. Ik stel voor dat we de 'longen' uitdagen voor een wedstrijdje zeilen, dat gaan we winnen!

De tijd voor het afronden van dit proefschrift ging verloren als werktijd op de poli. Vandaar dat ik mijn collega's Sanne, Bibi, Linda, Feiko, Nicole, Suzanne, Serge, Sander, Audrey, Ines, Marja, Mayke H, Mayke K, Dirk Jan, Kim, Maurice, Dorothee en Marlies, heel erg wil bedanken voor jullie inzet tijdens mijn afwezigheid. Ines en Suzanne, vooral jullie bedankt voor de moeilijke roosters om waar nodig mij vrij te plannen. Vanaf 1 november ben ik voor 110% inzetbaar! En Kim, de volgende keer dat we elkaar na sluitingstijd tegenkomen, gaan we in de Brink eten. Of liever een biertje in de Basket?

Discussie

Voor zeilen heb je niet alleen een boot met goede stuurlui en bemanning nodig. Met een beetje wind, goeie zin en een blik op de horizon gaat het pas echt lekker…

De thuishaven in het WKZ was dé flexkamer. Het was een leuk idee om werk en privé gescheiden te houden, maar als je 8 uur per dag bij elkaar op de kamer zit ben je volledig op de hoogte van elkaars lief en leed. Alleen over jullie kan ik al pagina's dankwoord vullen. De eerste jaren was ik matroos bij zeebonken Yvette, Arieke en Marijke, dames met levenservaring en praktische (statistische) kennis van zaken. Stefan zat als enige man op de andere kamer te filosoferen met Bar over allochtonen in Nederland. Daarna kwam Nienke, onze vliegensvlugge ex-Belg op de vouwfiets. Jojo danste ook even langs, daarna werd Coralie mijn vaste buurvrouw om mee te kletsen en een rondje te joggen. Brita, nóg een ex-Belg, met zeilervaring. Klasse dat je nu in Rotterdam je thuishaven hebt! Jopje, de kleine kapitein, je hebt een groot hart. Daarmee wordt het eindresultaat vast fantastisch. De Mariekes, de één in zichzelf pratend en met krullen, de ander met steil haar en bijdehand. Beiden blond en goedlachs. Evenals Bas, met gespitste oren als er ergens gefluisterd wordt. Alsof jullie nog niet genoeg waren, sloten Martijn en Berber van poli paars aan bij dit gezellige kliekje. Helaas is het kookclubje na twee keer in het water gevallen, maar laten we de traditie van het jaarlijkse weekendje niet verwateren!
Caro, jij hoort ook bij de flexers. Met jou heb ik de langste tijd op de flexkamer doorgebracht. In

die periode hebben we allebei stormen doorstaan. Soms waren de golven zo hoog dat je de horizon even niet kon zien, maar gelukkig hebben we nu allebei een rustig baaitje gevonden. Met een glas prosecco zullen we als elkaars paranimfen toosten op de goede afloop!

Promoveren is leuk, maar af en toe moet je de bakens verzetten. Een vrouwenavond met thee en koekjes en Net5 op de Justus is heerlijk, hoewel gemengd wonen uiteindelijk het gezelligst is. Even lekker buitengaats met de Guusjes. Tegenwoordig allemaal goede zeilers, dat kleine zeil is nu de fok en die irritant klapperende balk de giek. Maris, durf je ooit nog eens bij mij aan boord? Oppassen voor lager wal, voor je het weet is het weer rietzeilen. Jetski, volgend jaar gaan we weer naar Friesland met jou als kapitein van de Beerenburgboot! Lu, zie je al land daarboven in de mast? Daar gaan we volgende keer heen.
Midden in de winter naar buiten? Ja hoor, met z'n vieren in een brakke oude auto op weg naar het studenten hockeyveld en later naar de bossen van Groenekan. Marijke, Iris en Kim, die tijd is (gelukkig?) voorbij. We zijn nu volwassen volgens mij, maar nog lang geen veterinnen!
Als ik van de toekomst droom Hanne, dan gaan we een échte stoere boot kopen en zeilen.
Aanmeren bij een leuk stadje betekent een dag lang de voorraad gadgets en kleren bijvullen. Lieve Ing, ik heb bewondering voor de manier waarop jij met Jochem en Taeke altijd weer tijd hebt kunnen maken om samen de stad in te gaan, overdag of 's nachts. Met een kop thee erbij kan ik met jou werkelijk alles bespreken. Op naar Madrid.
Voor mijn broer Marijn was ik altijd de hippie, maar dat komt uit een goed hart en met een knipoog. Stiekem ben ik behoorlijk trots op je, grote broer! Eefje, jij bent m'n allerleukste zussie! Samen zijn we naar NYC gegaan, waar jij met je ruimtelijk inzicht een goeie gids was en we tenminste nog iets cultureels van de stad hebben gezien. Heel fijn dat jouw scriptie nu ook af is. Een dikke lebberzoen. Jansen, weet je de weg naar de SOA poli nog? Gewoon even vragen, luid en duidelijk. Je bent altijd welkom om een bakkie koffie te drinken tussen het studeren door. Je bent m'n kleine broer en paranimf. Je had gelijk; het boekje is prachtig geworden! Alle lof aan Rogier en neef Hendrik, supermooi werk.

Tot slot, lieve Martijn, jij bent natuurlijk m'n rots in de branding. Betrouwbaar, lief, stoer, handig, gezellig en je hebt lekkere krullen. Dus eigenlijk heb ik je liever aan boord, om samen weer het water in te springen!

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Appendices

List of abbreviations

B.	bifidobacterium
CPE	crude peanut extract
CFU	colony forming units
DBPCFC	double-blind placebo-controlled food challenge
ED	eliciting dose
HC	healthy control
IgE	immunoglobulin E
IU/ml	international units per milliliter
kU/L	kilo units per liter
L.	lactobacillus
Lc.	lactococcus
LTP	lipid transfer protein
MIA	micro-array immunoassay
N	number
NA	non-atopic
NOAEL	no-observed-adverse-effect level
OAS	oral allergy syndrome
PA	peanut-allergic
PS	peanut-sensitized non-allergic
RAST	radio-allergosorbent test
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SI	stimulation index
SNR	signal-to-noise ratio
SPT	skin prick test
STAI	State-Trait Anxiety Inventory

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Curriculum Vitae

Anne Elisabeth Flinterman is geboren op 10 november 1975 te Leusden. Na het behalen van haar cum laude diploma aan het Johan van Oldenbarnevelt Gymnasium in Amersfoort begon zij direct daarna, in 1994, aan de studie geneeskunde aan de Universiteit van Utrecht. Tijdens wetenschappelijke stages participeerde zij in onderzoek op de afdeling neonatologie in het Wilhelmina Kinderziekenhuis te Utrecht (het effect van hypoxie op het brein in aterme neonaten) en bij het Minneapolis Medical Research Foundation, Minnesota in de Verenigde Staten (infectie met cytomegalovirus van macrofagen en microglia). De co-schappen neurologie en KNO werden tevens in het buitenland doorgebracht, respectievelijk in Melbourne en Dublin. Het artsexamen werd behaald in oktober 2001.

Aansluitend is zij in november 2001 als arts-onderzoeker begonnen op de afdeling Dermatologie en Allergologie onder begeleiding van Prof. Dr. C. Bruijnzeel, Dr. S. Pasmans, Dr. A. Knulst, Dr. M. Hoekstra, Dr. E. van Hoffen en Dr. E. Knol. Voor een deel van dit onderzoek is zij korte tijd in het laboratorium bij Dr. Wayne Shreffler en Prof. Hugh Sampson in New York geweest. De resultaten van het gehele promotie-onderzoek worden in dit proefschrift besproken. Vanaf januari 2006 is ze de opleiding tot dermatoloog gestart met Prof. Dr. C. Bruijnzeel-Koomen als opleider.

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