5

Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy?

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Abstract

Background
Recognition of specific peanut allergens or the diversity of IgE binding to peanut allergens may play a role in the elicitation of severe allergic reactions.

Objective
To investigate whether sensitization to individual allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 is correlated with clinical severity.

Methods
The reactivity of purified peanut allergens was measured by skin prick test (SPT) and by IgE-immunoblot in 30 patients. The results were related to the clinical reactivity by history, and in 25 of them to the eliciting dose (ED).

Results
The majority of patients recognized Ara h 2 and Ara h 6. Patients with severe symptoms had a higher SPT response to Ara h 2 and Ara h 6 at low concentrations (0.1 μg/mL) and to Ara h 1 and Ara h 3 at higher concentrations (100 μg/mL), compared to patients with mild symptoms. They also recognized a greater number of allergens and showed a higher cumulative SPT response compared to patients with mild symptoms. No significant differences were observed between patients with a low or high ED.

Conclusions
Ara h 2 and Ara h 6 appeared to be more potent than Ara h 1 and Ara h 3. Both SPT reactivity to low concentrations of Ara h 2 and Ara h 6 and to higher concentrations of Ara h 1 and Ara h 3 were shown to be indicative of severe symptoms.

Introduction

Peanut allergy is a significant health problem because of its high and rising prevalence, its persistence, and the life-threatening nature of reaction.[1,2] In developed countries, it affects about 0.4-0.6% of children and 0.3-0.7% of adults.[3,4] Doses as low as 100 µg of peanut protein have been shown to elicit subjective allergic reactions.[5] Peanut allergy tends to be more severe in nature than other food allergies and accounts for 63% of the fatalities due to anaphylactic reactions to food.[6,7]

Several proteins have been identified as peanut allergens, characterized and subsequently designated Ara h 1 to Ara h 8. Historically, it was believed that Ara h 1 and Ara h 2 were the most relevant peanut allergens,[8-11] but a role for Ara h 3 and Ara h 6 could not be excluded. Ara h 1 and Ara h 2 are classified as major allergens, recognized by 70-90% of sensitized subjects.[9,11,12] Ara h 1 belongs to the vicilin family, and Ara h 2 to the conglutin family, which is related to the 2S albumin superfamily of seed storage proteins.[13] The peanut glycinin Ara h 3 is regarded as a minor allergen, but recently it was found that a group of peanut allergic Italian children were specifically sensitized to the basic subunit of Ara h 3.[14] Ara h 6 shows homology to Ara h 2.[10,15,16] In a recent study from our group it was shown that peanut-allergic patients recognize Ara h 6 both *in vitro* and *in vivo* to a similar extent as to that of Ara h 2,[10] indicating that Ara h 6 should be considered a major peanut allergen as well.

For some allergenic plant foods it has been shown that sensitization to a specific allergen is associated with severe allergic reactions. For example, sensitization to non-pollen-related hazelnut allergen Cor a 8 (lipid transfer protein, LTP) can be related to severe reactions to hazelnut,[17,18] whereas sensitization to the Bet v 1-related Cor a 1 is almost exclusively associated with oral allergy symptoms. For peanut, the association between sensitization to certain peanut allergens and the severity of reactions seems less clear. Lewis et al[19] showed that diversity of IgE binding to Ara h 1, Ara h 2 and Ara h 3 as determined by Western blotting was more important than the recognition of individual proteins. Shreffler et al[20,21] reported that patients with IgE antibody binding to multiple epitopes of Ara h 1, Ara h 2 and Ara h 3 tend to have more severe allergic reactions compared to those with IgE specific to a relatively few epitopes. Together, these data suggest that IgE binding to a greater number of allergens plays a more important role than specific allergen recognition does. However, preliminary data from our group indicate that specific recognition may play a role as well.[9]

To gain insight into the reactivity of the individual major allergens, the current work focuses on the reactivity of purified Ara h 1, Ara h 2, Ara h 3 and Ara h 6 *in vivo* by skin prick test (SPT) using tenfold dilutions, and *in vitro* by IgE-immunoblot. To investigate whether sensitization to (one of) these allergens can predict clinically severe reactions to peanut, the results were related to the clinical reactivity by history

and by eliciting dose (ED) determined by double-blind placebo-controlled food challenge (DBPCFC).

Materials and Methods

Study population

Thirty-one adult patients from the outpatient clinic of the Department of Dermatology/Allergology of the University Medical Center Utrecht were selected. Inclusion criteria were a history of allergic reactions to peanut in the patient's medical record, in addition to a positive SPT to peanut extract (ALK-Abelló, Nieuwegein, The Netherlands) with the area of the peanut wheal at least half of the area of the positive control (SPT ≥2+), and/or specific IgE to peanut ≥0.7 kU/L (CAP-FEIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).
Pregnancy, significant concurrent disease, instable asthma and oral medication with corticosteroids or ß-blocking agents were exclusion criteria.
This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All patients gave written informed consent before enrolment in the study.

Clinical evaluation

The subsequent step comprised a careful and complete medical history, SPT and blood sampling. One patient (P01KP) was excluded, because her reported allergic reaction did not appear to be caused by peanut. Symptoms by history were classified according to Muller, a scoring system which was originally designed for the classification of allergic reactions to insect venom.[22] Symptoms of the oral cavity were classified as Muller grade 0, symptoms of the skin and mucous membranes (urticaria, angioedema, rhinitis, conjunctivitis) as grade 1, gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain) as grade 2, respiratory symptoms (asthma, hoarseness) as grade 3, and cardiovascular symptoms as grade 4.
Specific IgE levels to peanut, and also to green pea, soy, grass pollen and birch pollen, were determined by the CAP system FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).
Clinical reactivity to peanut was investigated by DBPCFC in 25 of the 30 patients according the threshold consensus protocol[23] with some modifications[24]. Five patients did not participate for various reasons: anxiety (n=2), too busy (n=2) or no interest (n=1).
The hospital pharmacy prepared the challenge materials. The amounts of peanut flour were 0.01 mg, 0.1 mg, 0.5 mg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g and 3 g. The peanut flour used was partially defatted, light roasted (protein content 50%, fat 12%),

provided by J. Nordlee, Food Allergy Research and Resource Program, University of Nebraska, Lincoln, NE, USA. To mask the peanut doses, wheat instant cereal-cinnamon mix and apple sauce were added. Four similarly prepared placebo doses were randomly interspersed between the increasing peanut doses.
The challenge was discontinued when objective symptoms occurred, or when convincing subjective symptoms had occurred for at least three times or lasted for more than 45 minutes. The eliciting dose (ED) was determined as the lowest dose of peanut flour inducing convincing subjective symptoms. If the DBPCFC was negative, the patient subsequently underwent an open provocation with 1 g, 3 g and then 10 g of roasted peanuts.
All the challenges were conducted in a hospital setting, with careful monitoring of the patients. Full emergency treatment was readily available.

Purification of Ara h 1, Ara h 2, Ara h 3 and Ara h 6

Previously developed purification protocols were used for the preparation of Ara h 1[25], Ara h 2[10], Ara h 3[26] and Ara h 6[10] with resulting purities of >95% as judged by SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining. Purified peanut allergens were stored and sterilized as described earlier.[9]

Skin Prick Tests

All 30 patients included in the study were evaluated by SPT with commercial extracts of peanut, green pea, soy, grass pollen and birch pollen (ALK-Abelló, Nieuwegein, The Netherlands) using 1 mm-tip lancets (ALK-Abelló, Nieuwegein, The Netherlands) on the flexor aspect of the forearm following the recommendations of the EAACI.[27] Histamine dihydrochloride (10 mg/mL) and saline served as positive and negative controls, respectively. In addition, patients were tested with purified Ara h 1, Ara h 2, Ara h 3 and Ara h 6. For optimal accuracy, SPT were performed in serial tenfold dilutions, ranging from 100 μg/mL to 0.01 μg/mL. Serial 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.
Antihistamines were discontinued 1 week prior to the skin tests, and no topical corticosteroids were allowed on the day of skin prick testing on the flexor aspect of the arm.
The SPT reactivity was measured after 15 minutes. SPT responses were expressed as the ratio of the wheal reaction in millimetres squared, evaluated by computer scanning[28], divided by the wheal reaction of the positive control.[29] SPT ratios were regarded positive when the ratios were ≥0.25, i.e. when the wheal area was at least 25% of the wheal induced by the positive control. All SPT were carried out by the same investigator. Ten non-peanut-allergic patients were used as negative controls for SPT with purified allergens. SPT responses in these control subjects were all negative.

SDS-PAGE and IgE-immunoblotting

SDS-PAGE and IgE-immunoblotting was performed using 15% acrylamide gels as described earlier.[9] Patients' reactivity towards the purified peanut allergens was analyzed using IgE-immunoblotting, generally as described earlier.[9] Patient serum was diluted 50 times, and IgE bound to the membrane was detected with a peroxidase-conjugated goat-anti-human IgE (Kirkegaard and Perry Limited, Gaithersburg, MD, USA). Non-specific binding of the anti-IgE antibody conjugate was negligible as demonstrated by immunoblotting with non-peanut-sensitized human serum.

Statistics

All analyses of data were performed with nonparametric tests. The Mann-Whitney U test was used for comparison between groups. Correlations were analysed with Spearman's rank, or Gamma test when the data contain many tied observations (Figure 4A). Calculations were performed using SPSS (version 12, SPSS Inc., 2001, Chicago, USA). P values <0.05 were considered statistically significant.

Results

Patient characteristics

Thirty patients (20 female and 10 male) with a convincing history of peanut allergy and sensitization to peanut entered the study. The mean age was 28 years (range, 16 to 70). Eight patients (27 %) reported mild symptoms as their most severe symptoms by history (Muller grade 0 and 1), 7 patients (23 %) reported moderate symptoms (grade 2), and 15 patients (50%) reported severe symptoms (grade 3 and 4). Patient characteristics are summarized in Table 1.

Fifteen patients (50%) suffered from concomitant asthma and 24 (80%) patients from atopic eczema, whereas 27 (90%) patients reported pollinosis symptoms. Sensitization to grass pollen was present in 97% of the study population and to birch pollen in 90%. Twenty-three patients (77%) were sensitized to soy and 20 patients (67%) to green pea.

Levels of IgE to peanut ranged from 0.4 to >100 kU/L. Peanut specific IgE in patients with severe symptoms (median 18.4 kU/L) was higher than in the patient groups with mild (median 4.5 kU/L) and moderate symptoms (median 2.1 kU/L) ($p=0.01$ and $p=0.02$, respectively), but there was no significant difference between patients with mild and moderate symptoms ($p=0.82$).

Table 1
Clinical characteristics and reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 by SPT and IgE-immunoblot.

Patient no	Sex	Age	Mul-ler*	CAP peanut (kU/L)	SPT peanut (ratio)	Subjective ED (mg)	Objective ED (mg)	Most severe symptoms during challenge at a dose (mg)°	Time interval (years) §	SPT (ratio)				IgE-immunoblot			
										Ara h 1	Ara h 2	Ara h 3	Ara h 6	Ara h 1	Ara h 2	Ara h 3	Ara h 6
P05KP	F	22	0	18	1.3	10	-	ap at 10	>5	0.25	1.80	0.34	1.98	+	++	+	++
P17KP	M	21	0	0.4	0.9	10	-	oas	<1	0.05	1.68	0.16	2.85	+	+	+	-
P21KP	F	21	0	0.4	1.4	10	-	oas	>5	0.14	0.75	0.03	1.53	+	+	+	-
P28KP	F	24	0	1.9	1.0	nt	-	nt	<1	0.00	0.00	0.00	0.22	-	-	-	-
P02KP	M	21	1	4.7	1.9	300	-	oas	1-5	0.15	1.59	0.00	0.39	-	++	-	+
P09KP	M	23	1	14	8.0	nt	-	nt	>5	0.42	1.98	0.00	3.71	+	++	-	++
P12KP	M	20	1	4.3	1.8	0.1	-	oas	<1	0.15	0.98	0.00	1.26	+	+	+	+
P20KP	F	35	1	16	1.4	1	300	rc at 300	>5	0.00	0.18	0.00	0.77	-	-	+	-
P07KP	M	32	2	1.8	2.4	100	-	n at 300 and ap at 1000	1-5	0.14	1.13	0.16	1.66	-	+	-	+
P08KP	F	24	2	9.2	2.8	nt	-	nt	<1	0.27	1.57	0.55	1.59	+	++	+	++
P13KP	F	39	2	2.1	4.6	nt	-	nt	1-5	0.49	1.02	0.30	1.27	-	+	+	+
P15KP	F	27	2	33	6.8	10	-	n at 300	1-5	0.31	4.42	0.49	4.17	-	++	+	++
P19KP	F	29	2	1.7	2.9	10	-	ap and n and d at 1000	1-5	0.39	1.75	0.12	3.75	-	+	-	+
P25KP	F	20	2	38	1.7	no	-	-	1-5	0.00	0.00	0.09	0.37	-	+	+	-
P29KP	F	56	2	0.8	0.6	no	-	-	1-5	0.05	0.00	0.00	0.00	-	+	-	-
P04KP	F	19	3	3.1	1.9	100	-	oas	>5	0.00	0.28	0.25	0.86	+	-	-	-
P06KP	F	37	3	12	3.8	10	10	dia at 10 and 100	>5	0.71	0.93	1.49	0.77	+	+	++	+
P14KP	F	24	3	9.2	0.6	10	-	ap and n at 300	<1	0.18	2.57	0.21	0.61	-	+	-	+
P16KP	F	23	3	>100	5.9	0.1	-	d at 10	1-5	1.79	4.13	0.74	3.55	+++	>+++	>+++	>+++
P18KP	F	30	3	41	1.3	nt	-	nt	1-5	0.16	1.29	0.13	0.24	-	+	-	-
P22KP	M	33	3	>100	6.0	1	-	ap at 100 and d at 1000	>5	1.42	0.63	0.77	1.94	>+++	>+++	>+++	>+++
P24KP	M	18	3	11	2.6	0.5	3000	ap and n at 10 and v at 3000	<1	0.21	0.74	0.08	0.48	+	+	+	+
P26KP	F	29	3	4.2	8.8	100	-	ap and n at 100	<1	0.56	2.37	0.58	2.65	+	+	-	+
P30KP	M	27	3	46	2.0	1	-	d and ap at 10	1-5	0.35	4.20	0.59	1.67	++	++	++	++

P31KP	F	70	3	7.9	0.7	no	-	-	1-5	0.10	0.15	0.10	0.22	-	+	-	+
P03KP	M	16	4	>100	1.9	100	1000	d at 1000	>5	1.07	2.21	1.31	1.73	+++	>+++	>+++	>+++
P10KP	M	25	4	44	6.4	0.1	300	n and v at 300	>5	1.36	0.81	1.32	3.07	+	+++	++	+++
P11KP	F	22	4	85	6.2	0.1	-	n at 0.1	1-5	2.19	3.25	0.55	3.05	++	> +++	+++	>+++
P23KP	F	24	4	18	4.2	10	1000	d and h at 1000	<1	1.54	1.53	0.54	2.56	+	++	+	++
P27KP	F	28	4	18	14.8	0.1	-	oas	<1	0.87	1.91	0.75	2.64	-	+	-	+

* Patients are categorized according to ascending Muller score (most severe symptoms by history): 0, symptoms of the oral cavity; 1, symptoms of the skin and mucous membranes; 2, gastro-intestinal symptoms; 3, respiratory symptoms; 4, cardiovascular symptoms.
° d, dyspnea; ap, abdominal pain; dia, diarrhea; n, nausea; v, vomiting; oas, oral allergy symptoms; rc, rhino conjunctivitis; h, hoarseness.
§ Time between most severe historical reaction and SPT.
no: no ED detectable
nt: not tested

Eliciting doses (ED) and No Observed Adverse Effect Level (NOAEL) as determined by DBPCFC

Positive DBPCFC confirmed the diagnosis of peanut allergy in 22/25 subjects (88%). Three patients did not respond during the DBPCFC, of which two (P25KP and P29KP) underwent an open provocation that was negative. These two patients had experienced their last clinical reaction to peanut less than one year before start of this study. So, although still sensitized to peanut, these patients were not longer peanut-allergic. The third patient (P31KP) did not proceed to the open challenge, because of intercurrent disease. Her last reported allergic reaction to peanut was six years before the DBPCFC.

All patients tolerated a dose of 0.01 mg peanut flour, so the No Observed Adverse Effect Level (NOAEL) for our patient group in this study was 0.01 mg.

The ED for subjective reactions varied from 0.1 mg up to 300 mg peanut, and for objective symptoms from 10 to 3000 mg. The ED for subjective symptoms was significantly lower than the ED for objective symptoms (p=0.001). Objective symptoms consisted of dyspnea (n=2), diarrhea (n=1), vomiting (n=2) and rhino conjunctivitis (n=1).

To study whether the subjective ED was associated with peanut-specific IgE (CAP-FEIA), patients were divided in a group with a high ED (consisting of the 3 highest ED of 10-300 mg) (n=13) and with a low ED (consisting of the 3 lowest ED of 0.1-1 mg) (n=9). The concentration of specific IgE in patients with a low ED (median 44 kU/L; interquartile range 16.4-85) was significantly higher than in patients with a high ED (median 4.7 kU/L; interquartile range 1.8-17.7) (p=0.018).

Reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 by SPT and IgE-immunoblot

All thirty patients were subjected to titrated SPT with Ara h 1, Ara h 2, Ara h 3 and Ara h 6. There was a clear dose-response relationship for all allergens tested (Figure 1). Ara h 2 and Ara h 6 had a higher SPT reactivity than Ara h 1 and Ara h 3. Concentrations of 0.1 μg/mL resulted in positive reactions (ratio ≥0.25) for Ara h 2 and Ara h 6. Ara h 1 reactivity was positive starting at 10 μg/mL, whereas the reactivity to Ara h 3 began at 100 μg/mL. Ara h 2 showed comparable reactivity to Ara h 6, suggesting a similar potency of these allergens *in vivo*. The SPT results for Ara 1, Ara h 2, Ara h 3 and Ara h 6 in the highest concentration tested (100 μg/mL) for individual patients are shown in Table 1. The responses to this test concentration were similar in size to the response to the commercial peanut extract (Table 1).

The majority of patients with a positive SPT was sensitized to Ara h 2 (25/30, 83%) and Ara h 6 (26/30, 87%). Sixteen patients (53%) were sensitized to Ara h 1 and fifteen patients (50%) to Ara h 3. All patients with a positive SPT to Ara h 1 and/or Ara h 3, were also sensitized to Ara h 2 and/or Ara h 6. Three patients did not

recognize any purified allergen (P28KP, P29KP, P31KP).
Sera of all 30 patients were used for IgE-immunoblot analysis. IgE binding to purified allergens were scored in five categories from – (negative) to >+++ (strongly positive). Figure 2 shows representative examples of IgE-immunoblot experiments illustrating different intensities of IgE binding. The scoring results are summarized in Table 1, and largely confirm the SPT results. The majority of the patients showed IgE binding to Ara h 2 and Ara h 6. This IgE binding in the majority of the cases was more intense than binding to Ara h 1 and Ara h 3. There was a significant correlation between recognition in IgE-immunoblotting and SPT reactivity on the level of individual allergens, i.e. allergens that were most reactive in SPT were also most reactive in IgE-immunoblotting (correlation coefficient (γ) Ara h 1, Ara h 2, Ara h 3, Ara h 6: 0.552, 0.523, 0.54, 0.547, respectively ($p<0.001$ for all allergens)).

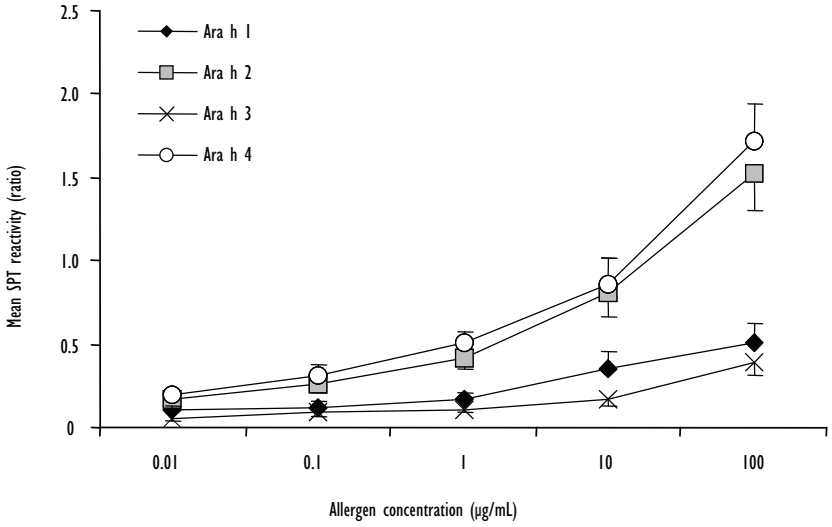

Figure 1
Dose response relationship of SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 (mean values ± SEM, n=30).

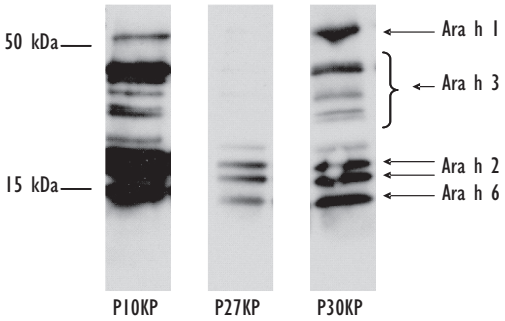

Figure 2
Examples of IgE-immunoblotting experiments with crude peanut extract (CPE). Patient sera (numbers indicated below blot lane) were incubated on blot membranes with SDS-PAGE separated CPE. Markers are shown left, and arrows indicate the positions of the individual peanut allergens.

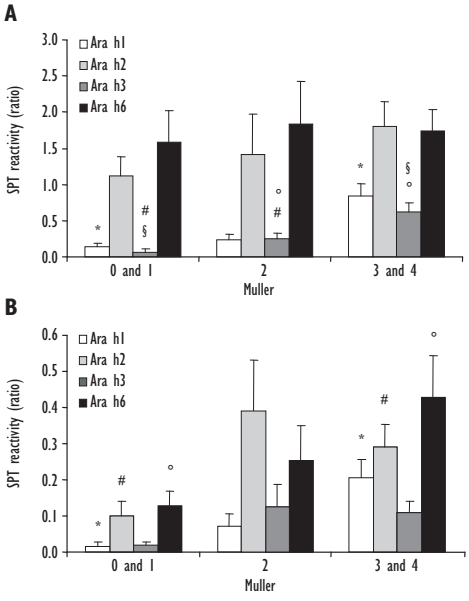

Figure 3
Sensitization to purified peanut allergens in different concentrations in relation to the most severe symptoms by history according to the Muller classification: (A) 100 µg/mL, (B) 0.1 µg/mL. *, #, §, and o: comparing two bars with the same symbols, p <0.05

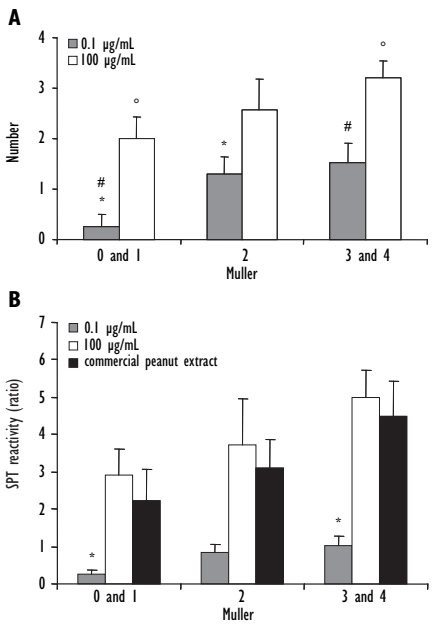

Figure 4
(A) Number of purified allergens recognized and (B) cumulative SPT score in relation to the reported symptoms by history according to the Muller classification.
*, #, and o: comparing two bars with the same symbols, p <0.05

Relationship between SPT reactivity to titrated purified allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 and the severity of peanut allergy

To assess whether the SPT reactivity to individual allergens was predictive for the clinical presentation of peanut allergy evaluated by history, the patients were categorized in Muller groups as described before (Figure 3).

The SPT responses to purified allergen concentrations of 100 μg/mL (Figure 3A) were higher for Ara h 2 and Ara h 6 than for Ara h 1 and Ara h 3 among all patient groups. The SPT reactivity to both Ara h 2 and Ara h 6 was not significantly different between the three groups. Patients with more severe symptoms after peanut intake had significantly higher SPT reactivity to Ara h 3 compared to patients with mild and moderate symptoms ($p=0.001$ and $p=0.048$, respectively). The reactivity to Ara h 1 was higher in patients with severe symptoms compared to patients with mild symptoms ($p=0.006$). SPT responses of purified allergens at lower concentrations (10-0.1 μg/mL) revealed smaller SPT responses. At all concentrations tested, the highest SPT responses were observed to Ara h 2 and Ara h 6 in the three patient groups. At 10 μg/mL, a significantly higher response to Ara h 1 and Ara h 3 was again observed in the group with severe symptoms compared to the group with mild symptoms ($p=0.041$ and $p=0.049$, respectively; data not shown). At lower concentrations, patients with severe symptoms had a significantly higher SPT reactivity to Ara h 1 (1 μg/mL (data not shown) and 0.1 μg/mL), Ara h 2 (0.1 μg/mL), and Ara h 6 (1 μg/mL (data not shown) and 0.1 μg/mL) compared to patients with mild symptoms (Figure 3B).

To assess whether clinical reactivity to peanut as demonstrated by ED was associated with the SPT reactivity, patients were categorized in two groups according to ED, as described before. At all concentrations used, there was no significant difference in SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 between the two groups. Only a trend to higher SPT responses in the group with a low ED was observed (data not shown).

Number of purified allergens recognized in SPT

Since the difference in symptom severity might be related to the total number of allergens recognized, we investigated whether patients with a history of more severe symptoms recognized a greater number of allergens than patients with mild symptoms. For the allergens tested at 100 μg/mL, a significantly greater number of allergens was recognized in the severe group compared to the patient group with mild symptoms ($p= 0.029$), whereas for 0.1 μg/mL, this difference was significant between the mild and moderate group ($p=0.033$) and between the mild and the severe group ($p=0.021$) (Figure 4A). No significant difference was found in the number of allergens recognized between the groups with a low or high ED for both concentrations tested (data not shown).

Cumulative SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6.
A relationship has been suggested between the level of specific IgE (SPT and CAP-FEIA) and clinical reactivity. Therefore, the sum of the SPT reactivities to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in relation to symptoms by history, and to ED was investigated (Figure 4B).
The cumulative SPT response of the purified allergens at a concentration of 0.1 μg/mL showed a significant difference between patients with mild and severe symptoms (p=0.002). Patients with more severe symptoms showed a trend to higher cumulative SPT response at a concentration of 100 μg/mL and also to a higher SPT reactivity to commercial peanut extract (p=0.155 and p=0.166, respectively).
Regarding the ED, no association between the cumulative SPT response and ED was observed (data not shown).

Discussion

The aim of our study was to investigate whether sensitization to the individual peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 was predictive for severe peanut allergy. Together, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 represent about three-quarters of the total protein content in peanut[30] and it is believed that these allergens are the most important ones in the peanut.[8-10,14]
The majority of all patients in this study recognized Ara h 2 and Ara h 6, both by SPT and IgE-immunoblotting. Immunoblotting evaluates allergen-specific IgE antibodies in serum on basis of direct binding to allergens separated by their molecular weight, whereas SPT is based on cross-linking of IgE molecules on the mast cells' IgE receptors. Our SPT data confirm our earlier reported data that Ara h 2 was more potent than Ara h 1 and Ara h 3.[9] This is in accordance with the study of Palmer et al[8], who found that Ara h 2 was a much more potent allergen than Ara h 1 using IgE-immunoblotting and a functional assay. We now additionally show that Ara h 6, which has structural similarities with Ara h 2[10], also has equal *in vitro* and *in vivo* potency.
To investigate whether sensitization to purified allergens was correlated with severity of symptoms, patients were categorized in three groups: mild (Muller grade 0 and 1), moderate (Muller grade 2) and severe (Muller grade 3 and 4). The group with severe symptoms had a significantly higher SPT response to Ara h 1 and Ara h 3 at a concentration of 100 μg/mL compared to the groups with milder symptoms (Figure 3). Lowering the test concentrations, Ara h 2 and Ara h 6 revealed also a significantly higher response in the group with severe symptoms compared to the other groups. In all three patient groups, the SPT reactivity to Ara h 2 and Ara h 6 was higher than the SPT responses to Ara h 1 and Ara h 3, demonstrating a higher allergenic potency

of Ara h 2 and Ara h 6 *in vivo*. The preferential recognition of Ara h 2 and Ara h 6 is not likely due to the allergen content of peanut, since the amount of Ara h 1 and Ara h 3 has been reported to be higher than Ara 2 and Ara h 6.[10,30] In summary, these data suggest that in general sensitization to Ara h 2 and Ara h 6, and in addition recognition of Ara h 1 and Ara h 3, are indicative for severe symptoms. Recently, Hourihane et al[31] devised a scoring system that combined the dosage that elicited the reaction and the clinical symptoms reported. Although it is a good concept to account for dose in a symptom scoring system, it is more difficult for patients (and investigators) to estimate the dose in milligrams of peanut protein that elicited the reaction than to describe clinical symptoms. Therefore, the patients were categorized using the Muller score.

In general, currently used commercial SPT extracts use high concentrations of allergens to reach a high sensitivity. However, our data show that differences in the patients' sensitivity to allergens may not be resolved when applied in saturating concentrations, as observed for Ara h 2 and Ara h 6. This suggests that the SPT response to allergens in very high concentrations may become positive in all sensitized patients, including patients with very mild or even without symptoms.

Recently, it was suggested that diversity of IgE binding to peanut epitopes[20] or allergens [19] is more important than the recognition of a specific allergen as determined by peptide-array and IgE-immunoblotting, respectively. To further investigate this, we determined the number of allergens that resulted in a positive SPT response in each patient (Figure 4A). We found that patients with histories of more severe symptoms did recognize a significantly greater number of allergens, in line with previous studies.[19,20,32] Furthermore, this was illustrated by the fact that a significantly higher cumulative SPT response was observed in patients with severe symptoms compared to patients with milder symptoms (Figure 4B). However, this was only detected at lower test concentrations, showing the importance of diluted purified allergens for SPT.

The allergic reactions to peanut by history were confirmed by DBPCFC in the large majority (88%) of the patients. The NOAEL in our study population was 0.01 mg peanut flour, corresponding to about 3/100,000 of a peanut. This is to our knowledge the first time that a NOAEL is described in peanut-allergic adults, according to the threshold consensus protocol. The lowest ED in our study was 0.1 mg peanut flour and this corresponds well with other reports.[5,33] We reported previously that patients with a history of severe symptoms had a significantly lower ED than patients with mild reactions.[5] This observation could not, however, be reproduced in this study. Hourihane et al[31] has theorized that prediction of future risk in the community may not be appropriate from the isolated result of DBPCFC, based on a poor correlation between the community- and challenge-based reactions and the effect of the fat content in the food on the ED. In addition, we could not find significant differences in SPT responses to the individual allergens, number of allergens recognized and

the cumulative SPT response between patients with a low ED and a high ED. This suggests that the ED does not reflect the severity of peanut allergy.
In conclusion, this study illustrates the relevance of SPT with diluted purified peanut allergens showing that the reactivity to all four allergens tested is correlated to the severity of peanut allergy by history. The observed difference in potency of Ara h 2 and Ara h 6 on the one hand and Ara h 1 and Ara h 3 on the other needs to be confirmed in oral challenge testing in order to further investigate the role of the individual peanut allergens in eliciting clinical reactions.

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