Monitoring peanut allergen in food products by measuring Ara h 1

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Background: Peanut allergy is an important health problem in the United States, affecting approximately 0.6% of children. Inadvertent exposure to peanut is a risk factor for life-threatening food-induced anaphylaxis.

Objective: The purpose of this investigation was to develop an immunoassay for a major peanut allergen, Ara h 1, to detect peanut allergen in foods so that the risk of inadvertent exposure can be reduced.

Methods: A specific 2-site monoclonal antibody–based ELISA was developed to measure Ara h 1 in foods. The sensitivity of the assay was 30 ng/mL. Ara h 1 was measured in foods (n = 83) with or without peanut and in experiments to optimize allergen yield and to determine peanut contamination in spiked foods.

Results: Ara h 1 levels in food products ranged from less than 0.1 μg/g to 500 μg/g. Ara h 1 measured in ng/mL was transformed to μg/g for food products. Peanut butter contained the highest amounts of Ara h 1. Peanut extracts contained from 0.5 to 15 mg Ara h 1/g of peanut depending on the extraction conditions. Optimal extraction of Ara h 1 was obtained by using phosphate buffer with 1 mol/L NaCl and Tween at 60°C. Ara h 1 was not always detected in presence of chocolate under the extraction conditions tested. Spiking experiments showed that the assay could detect ~ 0.1% Ara h 1 contamination of food with ground peanut. There was an excellent correlation between Ara h 1 levels and peanut content measured by using a commercial polyclonal antibody–based ELISA (r = 93, n = 31, P < .001).

Conclusion: A new sensitive and specific monoclonal antibody–based ELISA was used to monitor Ara h 1 content in food products. This assay should be useful for monitoring peanut contamination in the food manufacturing and processing industry and in developing thresholds for sensitization or allergic reaction in persons with peanut allergy. (J Allergy Clin Immunol 2003;111:640-5.)

Key words: *Peanut allergen, food allergy, immunoassay, environmental test*

Abbreviations used

1% BSA PBS-T: Phosphate-buffered saline containing 0.05% Tween-20 and 1% BSA
ppm: Parts per million
RT: Room temperature
SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Among the foods associated with hypersensitivity reactions, peanuts are the most frequently associated with severe reactions, including life-threatening food-induced anaphylaxis. Peanut allergy affects approximately 0.5% to 0.7% of children and in most cases persists throughout adult life.[1-7] Peanut (*Arachis hypogaea*) consumption is common in America, and the main allergens are preserved by dry roasting processes used in the preparation of peanut products.[8] Inadvertent exposure to peanut allergens, as part of the food ingredients or as a result of peanut contamination in food processing lines, is a major risk for patients with peanut allergy.

Enzyme immunoassays (ELISA) have been used in the food industry to detect peanut (and other foods such as hazelnut, almond, and egg) in manufacturing processes and in food products.[9-13] Typically, these assays use polyclonal antibodies directed against food extracts and measure antigenic components in parts per million (ppm) by comparison with a standard extract. Peanut allergens have been measured in foods by immunoassay with human IgE antibodies.[14] Although these assays have provided a useful generic screening tool, they are not allergen specific and cannot be used to provide quantitative measurements of exposure to food allergens. Recently, mAb assays have been developed to measure specific food allergens such as the major bovine allergen Bos d 2, soybean allergens, and the shrimp allergen Pen a 1.[15-18] The aim of the present study was to develop a mAb-based ELISA for a major peanut allergen (Ara h 1) and to compare Ara h 1 levels in foods.

Ara h 1 is a 65-kd glycoprotein that comprises 12% to 16% of the total protein in peanut extracts and causes sensitization in up to 95% of patients with peanut allergy.[19-24] Ara h 1 has a stable trimeric structure that protects IgE binding epitopes from degradation, and its allergenicity is preserved during food processing.[25-28] Here we show that Ara h 1 ELISA is specific and provides quantitative measurements of allergen levels in food products (either nanogram or microgram allergen per gram food). Ara h 1 is a useful marker for monitoring peanut contamination of food products.

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Supported in part by the Food Allergy Initiative, New York and by the NIH grant AI01666-04.

Presented in part at the American Academy of Allergy Asthma and Immunology Annual Meeting, March 2002.

Received for publication July 16, 2002; revised October 29, 2002; accepted for publication November 7, 2002.

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0091-6749/2003 $30.00 + 0

doi:10.1067/mai.2003.118

METHODS

Purification of Ara h 1

Ara h 1 was purified from defatted Florunner peanut extract by ammonium sulfate precipitation and cation exchange chromatography.[25] Purified Ara h 1 showed a major band at 63 kd on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein content was determined by absorbance at 280 nm by using the formula $A_{280} = 1.75 = 1$ mg/mL. Purified Ara h 1 was used for mAb production and as a standard for ELISA.

Monoclonal antibodies against Ara h 1

Ara h 1 specific mAbs were produced at the Lymphocyte Culture Center (University of Virginia, Charlottesville, Va) by using a standard protocol.[29,30] Four Balb/c mice were immunized by subcutaneous and intraperitoneal routes with 100 μg Ara h 1 in CFA at 4-week intervals. Approximately 5 months later, the selected mouse received intrasplenic and intraperitoneal injections of 10 μg Ara h 1, and 4 days later the spleen cells were fused with SP2/0-Ag14 myeloma cells.[29] Supernatants were screened for specific antibody production by ELISA. Antibody positive hybrids were cloned twice by limiting dilution, and frozen cell stocks were stored in liquid nitrogen. Four IgG1 anti-Ara h 1 mAbs (from a total of 35) were selected for ELISA development: clones 2C11-F12-D11 (2C11), 2F7-C12-D10 (2F7), 2C12-A11-A3 (2C12), and 2F8-G2-A12 (2F8). These mAbs were produced either as ascites or in gas permeable tissue culture bags (VectraCell; BioVectra, Charlottetown, Prince Edward Island, Canada) and purified by affinity chromatography over recombinant Protein G (Gamma Bind Plus; Pharmacia, Piscataway, NJ).

Two-site mAb ELISA

The assay was modified from a previously described ELISA for mite allergens.[31] Serial dilutions of mAb ascites (10^{-1} to 10^{-6}) were tested for binding to Ara h 1 in ELISA, and 2 mAbs (2F7 and 2F8) were biotinylated. The optimal mAb combination was established by comparing titration curves of Ara h 1 (0.002 to 1 μg/mL) by using the 4 capture mAbs at 1 μg/well and the biotinylated 2F7 and 2F8 mAbs at 1/1000 dilution for detection. The combination with capture mAb 2C12 and biotinylated 2F7 gave optimal binding. Microtiter plates were coated with 200 ng/well of mAb 2C12 in 0.05 mol/L carbonate-bicarbonate buffer, pH 9.6 overnight at 4°C and washed and incubated with PBS containing 0.05% Tween-20 and 1% BSA (1% BSA PBS-T) for 30 minutes. Plates were incubated with food extracts (dilutions from 1 to 1:1000) or with Ara h 1 standard for 1 hour. After washing, bound allergen was detected by using biotinylated mAb 2F7 (1:30,000 dilution for 1 hour) followed by streptavidin-peroxidase (100 μL at 0.25 μg/mL, 30 minutes). The color was developed by using ABTS (2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate (Sigma, St Louis, Mo), and plates were read after 10 minutes at 405 nm by using a spectrophotometer. The assay was quantitated by using a standard curve of doubling dilutions of purified Ara h 1 from 0.008 to 4 μg/mL. Values for food samples were interpolated from the linear part of the curve and expressed either as ng (or μg)/mL or as ng (or μg) Ara h 1 per g of food. Because the samples were obtained by extraction of 3 g of food in 30 mL of buffer, the ng/mL values were transformed to ng/g of food product by multiplying per 10.

Polyclonal antibody–based ELISA for peanut components

The peanut content of foods was also measured with a commercially available ELISA that uses rabbit polyclonal antibodies against peanut extract (Veratox; Neogen Corporation, Lansing, Mich). Results were expressed in ppm (2.5, 5, 10, and 25) of an extract prepared from 5 g of peanut in 125 mL of buffer (40 mg/mL) to form a standard curve.

Extraction of food products

Finished foods (n = 83) were purchased from local supermarkets and classified into 2 groups: peanut products including peanut cookies, peanut butter sandwich cookies, peanut sweets, and peanut butter and non-peanut products including cookies and a group of nuts, beans, and seeds. Peanut sweets include products such as peanut with chocolate bars, peanut and chocolate candies, peanut-caramel bars, butterfingers, wafers with peanut butter and chocolate, peanut butter cups, and peanut brittle. Chocolate-containing foods include a group of products from different categories; they may be peanut cookies, fudge sticks of peanut butter sandwich cookies, or peanut sweets. Food (3 g) was ground in a mortar and pestle and extracted with PBS by rocking for 2.5 hours at room temperature. Extracts were vortexed approximately every 20 minutes. The extract was centrifuged at 20,000*g* for 30 minutes, and the supernatant was centrifuged again for 5 minutes at 14,000*g* before being assayed or stored at –20°C.

Spiking experiments

Known amounts of peanut (2, 1, 0.5, 0.1, 0.025, and 0.006 g) were extracted in the presence of other food components up to a total of 3 g with 30 mL of buffer. The food components tested were non-peanut cookie and pancake mix. Controls with peanut alone (3, 2, 1, 0.5, 0.1, 0.025, and 0.006 g) were also extracted. Multiple extraction conditions were tested involving the use of 3 different buffers (PBS, Tris 20 mmol/L pH 7.5, and 20 mmol/L Tris pH 4.5), with and without 1 mol/L NaCl, and with different Tween percentages (0% to 2%). The highest Ara h 1 yield was obtained by using PBS with 1 mol/L NaCl and 2% Tween. Extractions were performed either at 60°C for 15 minutes or at room temperature (RT) for 2.5 hours.

RESULTS

Development of a mAb-based ELISA for Ara h 1

The mAb showed parallel binding curves in direct ELISA with Ara h 1 coated plates and had ascites titers of $\geq 1 \times 10^6$ (Fig 1, *A*). The mAbs with higher titer, 2F7 and 2F8, were biotinylated and were compared for binding to Ara h 1 with 4 different capture mAbs. The highest OD values were obtained by using capture mAb 2C12 and biotin 2F7, and this combination was selected for use in ELISA (Fig 1, *B*). The interassay variation was determined by assaying 50 extracts 3 times, and the intra-assay variation was established by measuring 10 extracts 3 times in the same assay. The interassay and intra-assay variations were 11.8% and 3.1%, respectively.

Detection of Ara h 1 in foods

Eighty-three products were extracted in PBS at RT and tested for Ara h 1 content. In general, dose response curves with various peanut-containing products were parallel to the Ara h 1 standard, and Ara h 1 values were extrapolated from the linear portion of the curve (Fig 2). Ara h 1 was detected at very low levels or undetected in peanut products containing chocolate. The products tested were peanut cookies (n = 10), peanut butter sandwich cookies (n = 7), peanut sweets (n = 9), and peanut butter (n = 12) and non-peanut foods including cookies (n = 18) and a group of beans, nuts, and seeds (n = 28). Given the

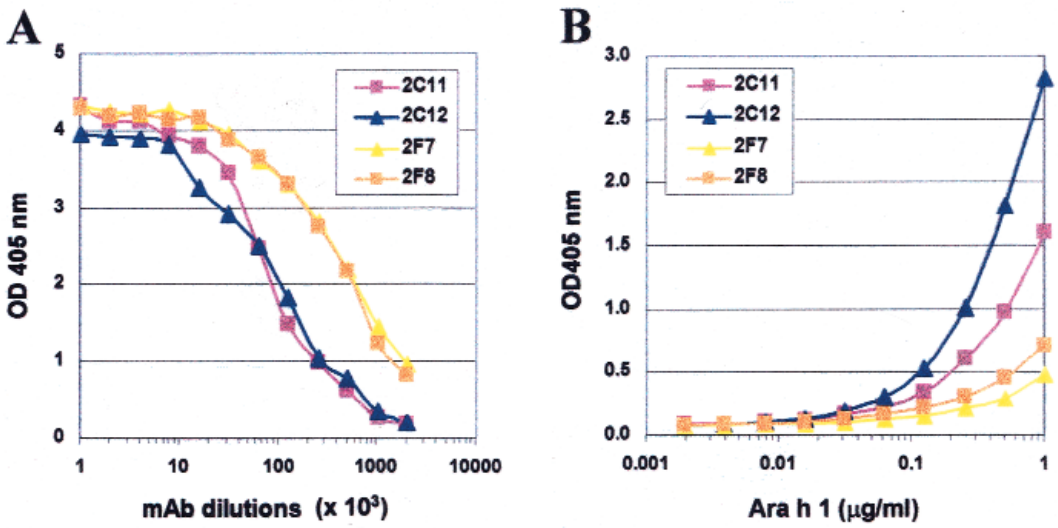

FIG 1. MAb binding to Ara h 1. MAb titration **(A)**: 4 mAbs in legend were tested for binding to Ara h 1 coating wells at 200 ng/well; and sandwich ELISA with biotinylated 2F7 with 4 different capture antibodies indicated in legend **(B)**.

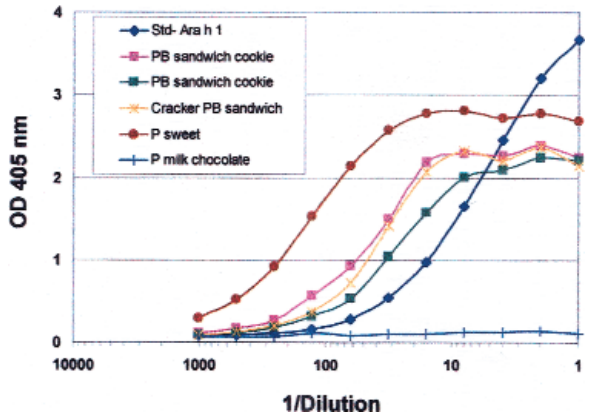

FIG 2. Comparison of Ara h 1 dose response curves using different products containing peanut *(P)* or peanut butter *(PB)*. Ara h 1 dilution curves of extracts from different peanut products. Ara h 1 curve starts at 4 µg/mL.

variety of products, there was a wide range of Ara h 1 concentrations from few ng/g to 500 µg/g of product. The geometric mean of Ara h 1 concentration for each of the mentioned groups was 11.5 µg/g for peanut cookies, 42.4 µg/g for peanut butter sandwich cookies, 11.4 µg/g for peanut sweets, 188.5 µg/g for peanut butter, and nondetectable levels for non-peanut cookies and beans, nuts, and seeds (Fig 3). The peanut products with lowest amounts of Ara h 1 contained chocolate or had very small proportion of peanut versus total product. Peanut extracts yielded the highest amount of Ara h 1 (0.5 to 15 mg/g).

Specificity of the Ara h 1 ELISA

Several tree nuts (almonds, brazil nuts, cashews, hazelnuts, pistachios, macadamia nuts, pine nuts, pecans, and walnuts), legumes (garbanzo beans, peas, kidney beans, soy beans, black-eyed beans, lentils, large and baby lima beans, navy beans, pinto beans, black beans, cranberry beans, great northern beans), sunflower seeds, and pearled barley were tested to study cross-reactivity of the Ara h 1 assay. Undetectable levels of Ara h 1 were measured in all the products (Fig 3).

Extraction conditions determine Ara h 1 yield

Optimal extraction conditions for Ara h 1 were investigated. Peanut ground to powder (3 g) was extracted by using buffer at 1:10 (w:v) (PBS or Tris), with different salt concentrations (0 mmol/L, 200 mmol/L, or 1 mol/L NaCl), temperature (RT or 60°C), and time (15 minutes and 2.5 hours). Ara h 1 content in peanut extracts ranged from 0.5 to 15 mg/g depending on the extraction conditions. The highest Ara h 1 yield, 15 mg/g, was obtained by using PBS with 1 mol/L NaCl at 60°C. High temperature increased Ara h 1 yield by 178% on average for the 6 extraction conditions. Tris 20 mmol/L without salt extracted more Ara h 1 (6.19 mg/g at RT and 6.63 mg/g at 60°C) than PBS without salt (0.56 mg/g at RT and 1.09 mg/g at 60°C). Ara h 1 was not detected in 2 samples of peanut oil that were extracted under the same conditions used for peanut extract.

Extraction of Ara h 1 in presence of food products

The goal of these experiments was to evaluate whether the Ara h 1 assay could be used to assess peanut contamination in food products. Food components (either ground non-peanut cookie or pancake mix flour) were spiked with known amounts of ground peanut (0.006 to 2 g), extracted by using different buffers, and assayed by ELISA. The highest Ara h 1 yield was obtained when extracting with PBS containing 2% Tween and 1 mol/L NaCl at 60°C for 15 minutes. These conditions significantly improved Ara h 1 yield when extracting 0.5 g of peanut (325 µg/mL at 60°C and 220 µg/mL at RT) com-

TABLE I. Spiking of foods to assess peanut contamination and Ara h 1 recovery

	Extracted for:	
	2.5 h at RT (μg/mL) (%)	15 min at 60°C (μg/mL) (%)
0.5 g peanut	220 (100)	325 (100)
0.5 g peanut in cookie	188 (86)	273 (84)
0.5 g peanut in pancake mix	231 (105)	307 (94)
0.1 g peanut	64 (100)	163 (100)
0.1 g peanut in cookie	11 (17)	26 (16)
0.1 g peanut in pancake mix	65 (102)	87 (54)
0.025 g peanut	18 (100)	32 (100)
0.025 g peanut in cookie	1 (8)	3 (8)
0.025 g peanut in pancake mix	12 (67)	20 (62)
0.006 g peanut	7 (100)	8 (100)
0.006 g peanut in cookie	0.4 (6)	0.5 (7)
0.006 g peanut in pancake mix	5 (66)	7 (86)

Ara h 1 recovered using different extraction conditions: Ground peanut (0.006-0.5 g) was extracted alone or with ground cookie or pancake mix made up to a total weight of 3 g. The food mix was extracted by using 30 mL of PBS with 1 mol/L NaCl and assayed for Ara h 1 by mAb ELISA.

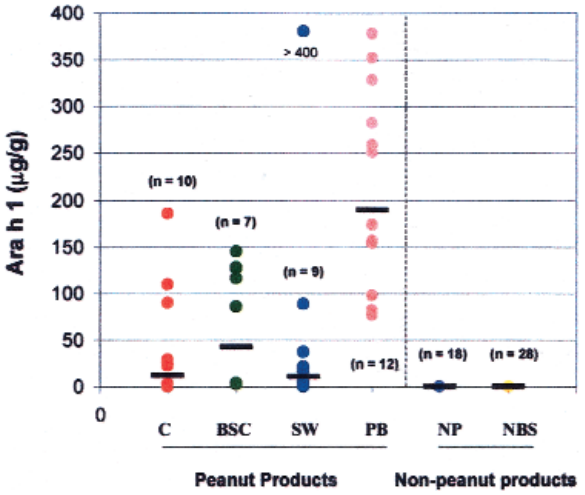

FIG 3. Ara h 1 levels in finished food products: peanut cookies *(C)*, peanut butter sandwich cookies *(SC)*, peanut sweets *(PS)*, peanut butter *(PB)*, non-peanut products *(NP)*, and nuts, beans, and seeds *(NBS)*.

pared with using PBS alone (13 μg/mL). The results showed that recovery of Ara h 1 progressively decreased when lower amounts of peanut were added to pancake mix, from 100% at 0.5 g peanut added to ~ 60% at 6 mg peanut (Table I). Lower yields were obtained by using cookie extract. However, for both cookie and pancake mix, Ara h 1 was detected in samples spiked with 6 mg of ground peanut (0.2% of product) (Table I). Extraction at 60°C increased recovery of Ara h 1. Only trace amounts of Ara h 1 were detected in chocolate that had been spiked with 0.5 g peanut, suggesting that components in chocolate bind the allergen (data not shown).

Comparison of the Ara h 1 ELISA and a polyclonal antibody–based ELISA for peanut components

Food products containing peanut were assayed by using both the Ara h 1 ELISA and a polyclonal antibody–based ELISA for peanut (Veratox). An excellent correlation was found between the Ara h 1 concentrations obtained with the Ara h 1 ELISA and the peanut components measured with the polyclonal antibody assay (r = 0.93, n = 31, $P < .001$; Fig 4). Most of the peanut food products (n = 31) contained detectable Ara h 1 and had positive values in the polyclonal ELISA. Two products (a peanut butter chocolate bar and a peanut chocolate candy) showed no detectable Ara h 1 but gave positive results in the polyclonal assay (775 and 38697 ppm, respectively). The Ara h 1 standard reacted weakly in the polyclonal ELISA with a sensitivity of 2 to 4 μg/mL, as compared to 30 ng/mL in the Ara h 1 ELISA (Fig 5). The polyclonal assay recognized 1 μg/mL of Ara h 2 as 11 ppm. The polyclonal antibody–based assay had high sensitivity for peanut in food, and some food extracts gave positive results at dilutions ≥ 1×10^5. However, the polyclonal ELISA had low sensitivity for Ara h 1 and Ara h 2.

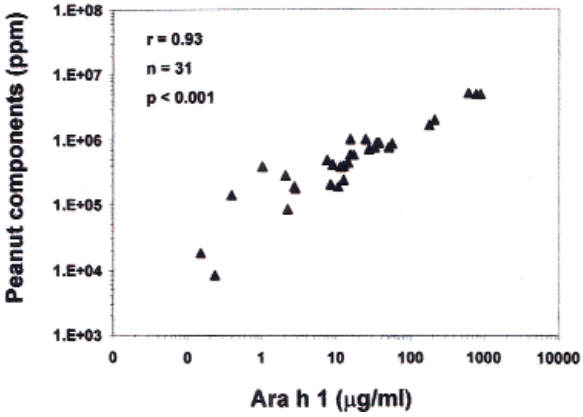

FIG 4. Correlation between Ara h 1 and ELISA for peanut components.

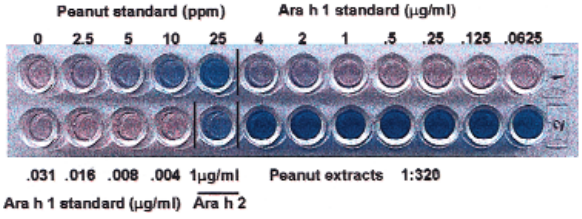

FIG 5. Polyclonal antibody–based ELISA for peanut components. Results show color intensities by using the commercial peanut standard (in ppm); the Ara h 1 standard (0.004-4 μg/mL); an Ara h 2 solution at 1 μg/mL; and 7 different peanut extracts containing from 500-15,000 μg/g Ara h 1 diluted 1:320. Color indicates lack *(pink)* or presence *(blue)* of peanut.

DISCUSSION

Panels of mAb were produced that show strong binding to Ara h 1 and were used to develop an ELISA. The assay was able to detect 30 ng/mL of Ara h 1 and was used to measure Ara h 1 levels in different food products. No significant cross-reactivity was observed between Ara h 1 and tree nuts, seeds, and especially legumes (Fabaceae or Leguminosae) that belong to the same botanical family as peanut. Although clinically important cross-reactivity to legumes in children is very rare, in vitro cross-reactivity is found among legumes, indicating that some proteins from different legumes have common epitopes.[32-35] For example, soybean glycinin G1 shares IgE epitopes with Ara h 3.[34] Ara h 1 shows 30% to 45% amino acid identity with other vicilin storage proteins from legumes such as soybean, garden pea, and beans. This degree of homology is usually enough to have similar molecular folding in homologous proteins; however, a higher degree of identity is usually required for high homology at surface residues. In keeping with this, the mAbs 2C12 and 2F7 showed no cross-reactivity between Ara h 1 and proteins produced by the 13 tested legumes.

Ara h 1 is one of the major peanut allergens and can be used as marker for peanut in foods. The mAb-based assay was used to measure Ara h 1 extracted from food products containing peanut. Extraction conditions greatly affected the Ara h 1 yield. Whereas some allergens such as patatin (Sol t 1) are heat-labile, Ara h 1 was readily extracted at 60°C, confirming that the Ara h 1 mAb-binding epitopes were heat stable at this temperature.[26,27,36] The highest Ara h 1 yield was obtained in the presence of salt and detergent at 60°C. These data are consistent with previous studies about the chemical properties of peanut proteins.[37] These conditions minimized the effect of food components on Ara h 1 extraction and measurement but did not completely avoid the inhibitory effect by chocolate, which is known to be a problem in food testing.[9,38] We have recently observed that Ara h 1 can be detected in chocolate by extraction in buffer with non-fat dry milk, and optimal conditions for measuring Ara h 1 in chocolate are being investigated.

The results show an excellent correlation between the mAb ELISA for Ara h 1 and a polyclonal ELISA for peanut components. Most of the peanut products analyzed were positive in both assays. The polyclonal ELISA detects multiple peanut components, and for peanut-positive samples most of the values were well above the standard curve. However, the polyclonal ELISA had low sensitivity for Ara h 1 and Ara h 2 allergens, indicating that this assay measures other peanut components. Our results support the view that polyclonal ELISA tests are useful tools for screening for peanut. The limitations of these assays are that the components being measured are not defined and the results cannot be accurately quantified. Expressing the results in ppm of a peanut extract may be acceptable for an individual manufacturer, but the unitage is arbitrary and the results cannot be directly compared with peanut tests produced by other manufacturers using different antisera and different peanut extracts. The advantages of measuring specific allergens, whether with mAb or polyclonal antibodies, are that the allergenic importance of these proteins has been defined and the results can be expressed in absolute value, ie, nanogram or microgram allergen per milliliter or per gram of food. These values can be compared between laboratories by using defined allergen standards. The WHO/IUIS Allergen Standardization Committee has established a program (CREATE) with the support of the European Union to develop international standards for purified inhaled allergens. Similar initiatives could result in the production of international standards for the major food allergens. Limitations of the Ara h 1 assay are that it measures only 1 allergen and the sensitivity is lower than for other mAb assays (30 ng/mL) (this could be increased by the use of amplification systems). These limitations could be overcome by developing assays for Ara h 2 and other peanut allergens.

Further studies are necessary to assess the clinical relevance of Ara h 1 concentrations in foods. The threshold dose for peanut exposure is variable and difficult to predict. The most sensitive individuals have been reported to respond to 0.2 to 2 mg of peanut protein.[5,39] However, the effective allergen concentrations that induce allergic reactions after ingesting a certain amount of peanut are unknown. The Ara h 1 assay will enable challenge studies to be performed with foods that contain known amounts of Ara h 1. We have also recently developed mAb to Ara h 2 and anticipate that assays for both major allergens will soon be available for comparative studies. These assays will be useful for investigating threshold doses of exposure and for risk assessment. There is increasing concern about inadvertent exposure of patients with allergy to foods, either as a result of allergen contamination of foods or mislabeling of food products.[40] The anti-Ara h 1 mAb has been used to develop a prototype rapid test that can detect Ara h 1 in 10 minutes. Such tests may be useful in the food industry for rapid monitoring of food products.

In conclusion, an mAb ELISA has been developed that can be used to monitor Ara h 1 levels in foods and allergen extracts. The mAb also provides excellent tools for measuring allergen-specific antibody responses and analyzing the antigenic structure of Ara h 1. The Ara h 1 assay will also allow investigation of how sensitization to peanut occurs and may provide better understanding of the mechanisms of peanut allergy.

We thank Dr Bill Sutherland and the Lymphocyte Culture Center for the production of the monoclonal antibodies against Ara h 1.

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