Epitope specificity of the major peanut allergen, *Ara h* II

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The antigenic and allergenic structure of Ara h *II, a major allergen of peanuts, was investigated with the use of four monoclonal antibodies obtained from BALB/c mice immunized with purified* Ara h *II. Our previous studies with monoclonal antibodies generated to peanut allergens showed this method to be useful for epitope mapping. When used as a solid phase in an ELISA, these monoclonal antibodies captured peanut antigen, which bound human IgE from patients with positive peanut challenge responses. The* Ara h *II monoclonal antibodies were found to be specific for peanut antigens when binding for other legumes was examined. In ELISA inhibition studies with the monoclonal antibodies, we identified two different antigenic sites on* Ara h *II. In similar studies with pooled human IgE serum from patients with positive challenge responses to peanuts, we identified two closely related IgE-binding epitopes. These characterized monoclonal antibodies to* Ara h *II will be useful for future studies to immunoaffinity purify the* Ara h *II allergen and to use in conjunction with recombinant technology for determining structure-function relationships. (J ALLERGY CLIN IMMUNOL 1995;95:607-11.)*

Key words: *Peanut, epitope specificity, monoclonal antibodies, IgE*

Peanuts are considered one of the most allergenic foods.[1] Peanut allergy is a significant health problem because of the potential severity of the allergic reaction, the chronicity of the allergic sensitivity, and the ubiquity of peanut products. Individuals sensitive to peanuts may experience symptoms ranging from mild urticaria to severe, systemic anaphylaxis.[1] In food-induced, fatal anaphylaxis, peanuts are the food most commonly implicated in causing the reaction.[2, 3] Sensitivity to peanuts often appears early in life, and unlike most other food allergies, tends to persist indefinitely.[4]

To elucidate the exact mechanism of IgE-mediated reactions, the identification and purification of the precise allergens are necessary. Significant information has accumulated in allergen characterization from a wide variety of sources, including pollens, dust mite, animal danders, and insects.[5] In comparison, allergen characterization for even the most common food allergens is much less defined. Despite the significant prevalence of peanut hypersensitivity reactions and several deaths annually, the identification of the clinically relevant antigens and an understanding of the immunobiology of peanut hypersensitivity is just beginning.

Abbreviation used
DBPCFC: Double-blind placebo-controlled food challenge

Monoclonal antibodies are being increasingly used to define and characterize the allergenic epitopes of many allergens. Multiple allergens including the dust mite allergen, *Der f* I,[6] and the grass pollen allergen, *Lol p* I,[7] have been studied by using monoclonal antibodies. Murine monoclonal antibodies to these allergens have been shown to be quite effective in defining their allergenic epitopes.

In this report we have investigated the epitope specificity of *Ara h* II,[8] a major peanut allergen, by using monoclonal antibodies as probes for mapping the possible antigenic determinants. We have produced and characterized a panel of monoclonal antibodies specific to *Ara h* II. The *Ara h* II monoclonal antibodies allowed us to define at least two

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antigenic sites on *Ara h* II. Inhibition assays were used to determine the IgE-binding sites on *Ara h* II.

METHODS

Patients with positive peanut challenge responses

Approval for this study was obtained from the Human Use Advisory Committee at the University of Arkansas for Medical Sciences. Twelve patients with atopic dermatitis and a positive immediate prick skin test response to peanut had either a positive response to double-blind placebo-controlled food challenge (DBPCFC) or a convincing history of peanut anaphylaxis (the allergic reaction was potentially life-threatening, that is with laryngeal edema, severe wheezing, and/or hypotension). Details of the challenge procedure and interpretation have been previously discussed.[9] Five milliliters of venous blood was drawn from each patient and allowed to clot, and the serum was collected. An equal volume of serum from each donor was mixed to prepare a peanut-specific IgE antibody pool.

Crude peanut extract

Three commercial lots of Southeastern Runners peanuts (*Arachis hypogaea*), medium grade, from the 1979 crop (North Carolina State University) were used in this study. The peanuts were stored in the freezer at −18° C until they were roasted. The three lots were combined in equal proportions and blended before defatting. The defatting process (defatted with hexane after roasting for 13 to 16 minutes at 163° C to 177° C) was done in the laboratory of Dr. Clyde Young (North Carolina State University). The powdered crude peanut was extracted in 1 mol/L NaCl, 20 mmol/L sodium phosphate (pH 7.0),[1] and 8 mol/L urea for 4 hours at 4° C. The extract was clarified by centrifugation at 20,000 *g* for 60 minutes at 4° C. The total protein determination was done by the bicinchoninic acid method (Pierce Laboratories, Rockville, Ill.).

Monoclonal antibodies

Mouse hybridoma cell lines were prepared by standard selection after polyethylene glycol–mediated cell fusion was carried out as previously described.[10] $Sp^2/0$-Ag14 mouse/myeloma cells were fused with immune splenocytes from female BALB/c mice hyperimmunized with *Ara h* II. Hybridoma cell supernatants were screened by ELISA and Western blotting, and cell lines were cloned by limiting dilution. The antibodies secreted by the monoclonal hybridoma cell lines were isotyped according the directions provided (Screen Type; Boehringer Mannheim, Indianapolis, Ind.). Ascites fluid produced in BALB/c mice was purified with Protein G Superose, as outlined by the manufacturer (Pharmacia, Uppsala, Sweden). Purified monoclonal antibodies were used in ELISA and ELISA inhibition assays.

ELISA for IgE

A biotin-avidin ELISA was developed to quantify IgE anti-peanut protein antibodies with modifications from an assay previously described.[11] The upper 2 rows of a 96-well microtiter plate (Gibco, Santa Clara, Calif.) were coated with 100 μl each of equal amounts (1 μg/ml) of anti-human IgE monoclonal antibodies, 7.12 and 4.15 (kindly provided by Dr. Andrew Saxon). The remainder of the plate was coated with the peanut protein at a concentration of 1 μg/ml in coating buffer (0.1 mol/L sodium carbonate–bicarbonate buffer, pH 9.6). The plate was incubated at 37° C for 1 hour and then washed five times with rinse buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20; Sigma Chemical Co., St. Louis, Mo.) immediately and between subsequent incubations. A secondary IgE reference standard was added to the upper 2 rows to generate a curve for IgE, ranging from 0.05 to 25 ng/ml.

The serum pool and patient serum samples were diluted (1:20 vol/vol) and dispensed into individual wells in the lower portion of the plate. After incubation for 1 hour at 37° C and washing, biotinylated, affinity-purified goat anti-human IgE (KPL, Gaithersburg, Md.) (1:1000 vol/vol bovine serum albumin) was added to all wells. Plates were incubated for 1 hour at 37° C and washed, and 100 μl horseradish peroxidase–avidin conjugate (Vector Laboratories, Burlingame, Calif.) was added for 5 minutes. After washing, the plates were developed by the addition of a citrate buffer containing o-phenylenediamine (Sigma Chemical Co.). The reaction was stopped by the addition of 100 μl 2N hydrochloric acid to each well, and absorbance was read at 490 nm (Bio-Rad Microplate reader model 450; Bio-Rad Laboratories Diagnostic Group, Hercules, Calif.). The standard curve was plotted on a log-logit scale by means of simple linear regression analysis, and values for the pooled serum and individual samples were read from the curve.[8, 9]

ELISA inhibition

An inhibition ELISA was developed to examine the site specificity of the monoclonal antibodies generated to *Ara h* II. One hundred microliters of *Ara h* II protein (1 mg/ml) was added to each well of a 96-well microtiter plate (Gibco) in coating buffer (carbonate buffer, pH 9.6) for 1 hour at 37° C. Next, 100 μl of differing concentrations (up to 1000-fold excess) of each of the monoclonal antibodies was added to each well for 1 hour at 37° C. After washing, a standard concentration of the biotinylated monoclonal antibody preparation was added for 1 hour at 37° C. The assay was developed by the addition of the avidin substrate as in the ELISA above.

A similar ELISA inhibition was performed with the peanut-positive serum IgE pool instead of the biotinylated monoclonal antibody to determine the ability of each monoclonal antibody to block specific IgE binding.

RESULTS

Hybridomas specific for *Ara h* II

Cell fusions between spleen cells obtained from female BALB/c mice immunized with *Ara h* II and the mouse myeloma cells resulted in a series of hybridomas specific for *Ara h* II. Seven monoclonal

TABLE I. Peanut-specific IgE to antigen presented by four monoclonal antibodies

Patient No.	Capture antibody			
	4996D6	4996C3	5048B3	4996D5
1	95%	80%	80%	91%
2	94%	66%	72%	90%
3	96%	114%	87%	96%
4	98%	116%	76%	96%
5	97%	74%	130%	107%
6	94%	63%	76%	86%
7	109%	123%	104%	116%
8	0%	0%	0%	0%
9	0%	0%	0%	0%

Ara h II monoclonal antibodies used as capture antibodies in ELISA with *Ara h* II as the antigen. Values are expressed as a percent of binding compared with challenge-positive peanut pool. Patients 1 to 7 had positive DBPCFC responses to peanut; patient 8 is the patient without peanut sensitivity with elevated serum IgE; patient 9 is the patient without peanut sensitivity with normal serum IgE.

TABLE II. IgE-specific binding to legumes captured by *Ara h* II monoclonal antibodies

	Capture antibody			
	4996D6	4996C3	5048B3	4996D5
Pooled serum*				
Ara h II (17 kd)	0.451	0.565	0.235	0.381
Crude peanut	0.137	0.409	0.161	0.170
Soy	0.053	0.055	0.055	0.015
Lima beans	0.033	0.026	0.029	0.025
Ovalbumin	0.028	0.029	0.029	0.035
Normal serum				
Ara h II (17 kd)	0.024	0.031	0.038	0.033
Crude peanut	0.017	0.027	0.028	0.024

Values are expressed as optical density units.
*Pooled serum is from patients with positive responses to peanut challenge.

antibody–producing lines were chosen for further study. In preliminary studies all seven hybridoma-secreting cell lines had antibodies that bound *Ara h* II, as determined by ELISA and immunoblot analysis.[12, 13] On the basis of different binding studies, four of the hybridomas were used for further analysis. As determined by isotype immunoglobulin-specific ELISA, all four hybridoma-secreting cell lines typed as IgG_1.

ELISA with monoclonal antibody as solid phase

Four monoclonal antibody preparations (4996D6, 4996C3, 5048B3, and 4996D5) were used as capture antibodies in an ELISA with *Ara h* II as the antigen. Serum from individual patients, who had positive challenge responses to peanut, was used to determine the amount of IgE binding to each peanut fraction captured by the *Ara h* II–specific monoclonal antibody (Table I). A reference peanut-positive serum pool was used as the control serum for 100% binding. Seven patients who had positive DBPCFC responses to peanut were chosen. All seven patients had significant amounts of anti-peanut–specific IgE to the peanut antigen presented by each of the four monoclonal antibodies compared with the control sera (patient 8 without peanut sensitivity who had elevated serum IgE values, patient 9 without peanut sensitivity who had normal serum IgE values). Titration curves were performed to show that limited amounts of antigen binding were not responsible for similar antibody binding. There were no significant differences in the levels of anti-peanut–specific IgE antibody to the peanut antigens presented by each monoclonal antibody. Most patients had their highest value for IgE binding to the peanut antigen presented by either 4996D6 or 4996C3, whereas no patient had his or her highest percent of IgE binding to the peanut antigen presented by monoclonal antibody 4996D5.

Food antigen specificity of monoclonal antibodies to *Ara h* II

To determine whether the *Ara h* II monoclonal antibodies would bind to only peanut antigen, an ELISA was developed with the pooled peanut-specific IgE from patients who had positive DBPCFC responses to peanut. All four monoclonal antibodies that were fully characterized bound only peanut antigen (Table II). In the ELISA no binding to soy, lima beans, or ovalbumin occurred. When the normal serum pool was used in the ELISA, no peanut-specific IgE to either *Ara h* II or crude peanut could be detected.

In the United States, three varieties of peanuts are commonly consumed: Virginia, Spanish, and Runner. In an ELISA, we attempted to determine whether there were differences in monoclonal antibody binding to the three varieties of peanuts. There was only a minor variation with the ability of the peanut-specific IgE to bind to the captured peanut antigen (data not shown).

Site specificity of four monoclonal antibodies

An inhibition ELISA was used to determine the site specificity of the four monoclonal antibodies to *Ara h* II (Table III). As determined by ELISA inhibition analysis, there are at least two different

TABLE III. ELISA inhibition for four monoclonal antibodies to *Ara h* II

	Inhibitory antibody				
	4996C3	**4996D6**	**5048B3**	**4996D5**	**Alt 1**
Biotinylated mAb					
4996C3	99%	8%	6%	3%	1%
4996D6	0%	53%	31%	18%	9%
5048B3	30%	83%	100%	100%	3%
4996D5	1%	44%	56%	64%	8%

Site specificity of four *Ara h* II monoclonal antibodies as determined by ELISA inhibition analysis. Values are expressed as percent inhibition.
mAb, Monoclonal antibody.

TABLE IV. Individual anti-peanut-specific IgE binding to *Ara h* II

	Serum dilution					
	1:320	**1:100**	**1:80**	**1:40**	**1:20**	**1:5**
4996D6	0%	0%	0%	0%	3%	5%
4996C3	14%	10%	10%	12%	10%	24%
5048B3	0%	5%	5%	5%	7%	11%
4996D5	0%	10%	10%	22%	23%	25%

Site specificity of four *Ara h* II monoclonal antibodies inhibiting anti-peanut-specific IgE (serum pool from patients with peanut hypersensitivity) binding to *Ara h* II. Values are expressed as percent of anti-peanut-specific IgE binding to *Ara h* II without inhibiting monoclonal antibody.

epitopes on *Ara h* II, which could be recognized by the various monoclonal antibodies (*epitope 1*—4996C3, *epitope 2*—4996D6, 5048B3, 4996D5). Seven different monoclonal antibodies generated to *Ara h* I, a 63.5 kd peanut allergen,[9] were used to inhibit the binding of the four *Ara h* II monoclonal antibodies to the *Ara h* II protein. None of the *Ara h* I monoclonal antibodies inhibited any binding of the *Ara h* II monoclonal antibodies.

Site specificity of peanut-specific human IgE

Results of inhibition assays with monoclonal antibodies to inhibit IgE binding from the IgE pool (from patients with peanut hypersensitivity) to *Ara h* II are shown in Table IV. Monoclonal antibodies 4996C3 and 4996D5 inhibited the peanut-specific IgE up to approximately 25%. Monoclonal antibodies 4996D6 and 5048B3 did not inhibit peanut-specific IgE binding. These two inhibition sites correspond with the two different IgG epitopes recognized by the monoclonal antibodies in the inhibition experiments.

DISCUSSION

The route of allergen administration, dosage, frequency of exposure, and genetic factors all determine the type and severity of an individual's allergic response.[14] To date, no distinct features, which would distinguish allergens as unique antigens, have been identified.[14] In contrast, only three foods in the United States (milk, eggs, and peanuts) account for approximately 80% of positive responses to food challenges in children.[15]

Although clinical sensitivity to most foods is typically lost as a patient ages, clinical sensitivity to peanut is rarely lost. For this reason, it is important to examine the peanut allergens to determine whether they have distinct features that would cause the persistence of clinical reactions.

Two major peanut allergens, *Ara h* I and *Ara h* II, have recently been identified and characterized.[8, 9] *Ara h* I has two major bands as determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis with a mean molecular weight of 63.5 kd and an isoelectric point of 4.55. *Ara h* II has a mean molecular weight of 17 kd and an isoelectric point of 5.2. Individual sequencing of *Ara h* I and *Ara h* II indicates that they are probably isoallergens.[8] Other peanut allergens have been identified including peanut 1[16] and concanavalin A–reactive glycoprotein.[17]

In this study four monoclonal antibodies to *Ara h* II were extensively characterized. All four monoclonal antibodies produced to *Ara h* II, when used as capture antibodies in an ELISA, presented antigens that bound IgE from patients with positive challenge responses to peanut. No significant differences were detected in the binding of IgE from any one patient to the allergen presented by the individual monoclonal antibodies. In separate ELISA experiments, the four monoclonal antibodies generated to *Ara h* II did not bind to other legume allergens and did not bind to one variety of peanuts preferentially.

To determine the epitope site specificity of these monoclonal antibodies, inhibition ELISAs were done. At least two different and distinct

IgG epitopes could be identified in experiments with the allergen, *Ara h* II. In related experiments done with pooled serum from patients with positive DBPCFC responses to peanut, two similar IgE epitopes were identified. The results of this study are comparable to those with monoclonal antibodies to *Der f* I[18] in which five nonoverlapping antigenic sites and three IgE-binding epitopes were identified. In our previous studies with *Ara h* I monoclonal antibodies,[19] four different antigenic sites were recognized, and three of these sites were IgE-binding epitopes.

In related experiments with other allergens, a variety of solid-phase inhibition assays have been used to block the polyclonal IgE response to the allergen being studied.[6] The interpretation of the level of inhibition that should be regarded as significant has varied from 15% to 80%.[6] The *Ara h* II monoclonal antibodies inhibited the polyclonal IgE response by up to 25%.

The characterization of these *Ara h* II monoclonal antibodies will allow future studies to better define the exact amino acid sequence that is responsible for IgE binding. Additionally, these monoclonal antibodies should make purification of the *Ara h* II allergen much simpler and more efficient. Immunoaffinity purification of allergens, such as that completed with the cockroach allergens[6] and with the *Ara h* I peanut allergen,[19] has produced a technique to purify allergens from a heterogeneous crude source material.

Future studies on the antigenic and allergenic structure of allergens will likely use monoclonal antibody techniques, in addition to recombinant DNA technology. Monoclonal antibodies will be used to map these epitopes and to identify cDNA clones specific for the allergens. Together, recombinant DNA technology and monoclonal antibody production will be used to examine the role of specific T-cell epitopes in the induction and regulation of the allergenic response.[20]

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