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Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges

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Peanuts and soybeans are two of the six most common foods to cause food hypersensitivity reactions in children. We used the serum of 10 patients with atopic dermatitis and positive double-blind, placebo-controlled, food challenges to peanut and two patients with atopic dermatitis and positive double-blind, placebo-controlled, food challenges to soybean to investigate the change in IgE-specific and IgG-specific binding to these proteins altered by either chemical or thermal denaturation. We used IgE- and IgG-specific ELISA-inhibition analyses to compare these effects on the crude peanut and crude soy extracts, as well as on the major allergenic fractions of both proteins. Heating the soy proteins at various temperatures and time intervals did not significantly change the IgE- or IgG-specific binding of the soy positive pooled serum. When the peanut proteins were subjected to similar heating experiments, the IgE- and IgG-specific binding did not change. When these same proteins were treated with enzymes in the immobilized digestive enzyme assay system used to mimic human digestion, the binding of IgE to the crude peanut and crude soy extracts was reduced; 100-fold for peanut and 10-fold for soybean. Therefore it appears that thermal denaturation of peanut and soybean protein extracts does not enhance or reduce IgE- and IgG-specific binding activity. Chemical denaturation appears to minimally reduce the binding of these proteins. (J ALLERGY CLIN IMMUNOL 1992;90:889-97.)

Key words: *Peanut allergens, soybeam allergens, food hypersensitivity, atopic dermatitis*

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Multiple allergens have been identified in the past several years that stimulate IgE-mediated disease in humans. The identification and purification of these allergens is essential for further studies to understand and characterize the immune response to these antigens.[1] Structural studies of these allergens is also critical to the understanding of the IgE-mediated response.[2] Several inhaled allergens have been characterized from a wide variety of sources, including dust mites, pollens, animal danders, insects, and fungi. Only recently have food allergens been studied with

Abbreviations used

IDEA: Immobilized digestive enzyme assay
DBPCFC: Double-blind placebo-controlled food challenge
ELISA: Enzyme-linked immunosorbent assay
pI: Isoelectric point
EDC: 1-ethyl-3-[3-(dimethylamino)propyl] carboimiide

similar techniques to further characterize this IgE-mediated response.[3-6]

The study of food hypersensitivity has been significantly advanced by the use of double-blind, placebo-controlled, food challenges (DBPCFCs) to document food hypersensitivity reactions.[7, 8] Children with atopic dermatitis and food hypersensitivity reactions have been particularly well studied. Approximately 60% of children with severe atopic dermatitis and 33% of children with less severe atopic dermatitis have been documented to have food hypersensitivity reactions after undergoing DBPCFCs.[9, 10]

The susceptibility of identified allergens to thermal or chemical denaturation has been previously studied with inhaled allergens such as house dust mite,[11] ragweed,[12] and rye grass.[13] Less is known about the alteration of the food allergens after similar treatment.[3]

We used the serum of 10 patients with atopic dermatitis and positive DBPCFCs to peanut and serum from two patients with atopic dermatitis and positive DBPCFCs to soybean to investigate allergen-specific IgE and IgG antibody by enzyme-linked immunosorbent assay (ELISA)-inhibition analysis. Significant changes did not occur after heating of either the peanut or soybean allergens. Moderate changes occurred after enzyme treatment of the same allergens. The results suggest the antigenic determinants of the major allergens in peanut and the allergenic fractions soybean are not readily denatured and are not conformation dependent.

MATERIAL AND METHODS

Food-sensitive patients

Approval for this study was obtained from the Human Use Advisory Committee at the University of Arkansas for Medical Sciences. Ten patients (mean age, 4.2 years) with atopic dermatitis and a positive immediate prick skin test to peanut had either a positive DBPCFC to peanut or a convincing history of peanut anaphylaxis (the allergic reaction was potentially life threatening, i.e., laryngeal edema, severe wheezing, and/or hypotension). Two patients (ages 3 and 16 years) with atopic dermatitis and a positive immediate prick skin test to soybean had a positive DBPCFC to soybean. Details of the challenge procedure and interpretation have been discussed previously.[10] Five milliliters of venous blood were 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 food-specific IgE antibody pool, one for peanut and one for soybean.

Crude peanut extract and fractions

Three commercial lots of southeastern runners *(Arachis hypogaea)* (Florunner), 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 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° 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) with the addition of 8 mol/L urea for 4 hours at 4° C.[14] The extract was clarified by centrifugation at 18,000 rpm for 60 minutes at 4° C. A 63.5 kd peanut fraction (tentatively designated *Ara h* I)[6] and a 17 kd fraction (tentatively designated *Ara h* II)[15] were purified from this crude peanut extract. In brief, a crude peanut extract was dialyzed against 20 mmol/L Tris-Bis-Propane (pH 7.2) and 8 mol/L urea, and 40 mg were loaded onto the anion exchange column (Mono Q, Pharmacia, Piscataway, N.J.) with use of the FPLC system (Pharmacia). The column elution was affected with a starting buffer of 20 mmol/L Tris-Bis-Propane/8 mol/L urea (pH 7.2) and a limit buffer of 1.5 mol/L NaCl/20 mmol/L Tris-Bis-Propane/8 mol/L urea (pH 7.2). The fraction eluting at 10% NaCl contained the 63.5 kd allergen.[6] The 17 kd fraction was isolated by use of a PL-SAX (anion exchange) column (Polymer Laboratories, Amherst, Mass.) under similar conditions. A crude peanut extract was dialyzed against 20 mmol/L Tris-Bis-Propane (pH 7.2) without urea, and 40 mg were loaded on the PL-SAX column and eluted as above without the addition of urea. The fraction eluting at 20% NaCl contained the 17 kd allergen.[15]

Crude soybean extract and fractions[16]

Commercial defatted soybean flakes were extracted (1:20 wt/vol) at room temperature for 45 minutes in deionized water (wt/vol) and adjusted to pH 8.0 with 1N NaOH.[17] After centrifugation at 35,000*g* for 20 minutes, the recovered supernatant represents the crude soy preparation. A whey preparation was obtained by adjusting the crude soybeam preparation to pH 4.0 with 1N HCl. The principal globulin proteins are recovered by centrifugation at 35,000*g* for 20 minutes. The supernatant (whey fraction) was recovered and adjusted to pH 7.0 with dilute alkali. The two major soy protein fractions, 7S and 11S, were isolated and purified according to a modification of the procedure of Thanh and Shibasaki.[17] Commercial defatted soy flakes were extracted (1:20 wt/vol) with 0.03 mol/L Tris-HCl buffer (pH 8.0) containing 0.001 mol/L dithioerythriotol at room temperature (20° to 22° C) for 1 hour. After centrifugation at 35,000*g* for 20 minutes (20° C), the supernatant was

recovered and adjusted to pH 6.4 with 1N HCl. A crude 11S protein precipitate was collected by centrifugation at 35,000*g* for 20 minutes. Purified 11S protein was obtained by resolubilizing the precipitate (1:20 wt/vol) in 0.035 mol/L phosphate buffer (pH 7.6) containing 0.4 mol/L NaCl, 0.001 mol/L dithioerythriotol, and 0.05% sodium azide and passed through a concanavalin A sepharose 4B column to remove contaminating glycoproteins. To obtain the 7S protein fraction, commercial defatted soy flakes were extracted (1:20 wt/vol) with 0.03 mol/L Tris-HCl buffer (pH 6.2) containing 0.001 mol/L dithioerythriotol at 9° C for 1 hour and centrifuged at 35,000*g* for 20 minutes (4° C). The supernatant was recovered, adjusted to pH 4.8 with 1N HCl, and centrifuged as above. The precipitate was diluted (1:10 wt/vol) with Tris-HCl buffer, adjusted to pH 7.6 with 1N NaOH and stirred at 9° C until the precipitate dissolved. The solution was readjusted to pH 6.2 and centrifuged. After the addition of 0.4 mol/L NaCl, the crude 7S supernatant was warmed to room temperature (20° to 22° C) and adsorbed to a concanavalin A sepharose 4B column preequilibrated with 0.03 mol/L Tris-HCl buffer (pH 6.2) containing 0.4 mol/L NaCl and 0.001 mol/L dithioerythriotol. After washing to remove the unbound proteins, purified 7S fraction was recovered by eluting the column with the same buffer containing 0.1 mol/L α-methyl-D-mannoside. The purified 7S solution was equilibrated against 0.035 mol/L phosphate buffer (pH 7.6) containing 0.4 mol/L NaCl, 0.001 mol/L dithioerythriotol, 0.05% sodium azide by repeated concentration (4x) in a stirred cell apparatus (Amicon Corp., Danvers, Mass.) containing a YM-10 membrane followed by redilution (1:10 vol/vol).

ELISA inhibition for IgE and IgG

A biotin-avidin competitive ELISA-inhibition analysis was done to compare antifood specific IgE antibodies with modifications from several assays previously published.[18, 19] The peanut and soybean products used in the inhibition studies were subjected to the following treatments: room temperature for 1 hour, 37° C for 1 hour, 56° C for 1 hour, 100° C for 5 minutes, 100° C for 20 minutes, and 100° C for 60 minutes. The upper two rows of a 96-well microtiter plate (Gibco, Santa Clara, Calif.) were coated with 100 μl each of equal amounts (1 μg/ml) of antihuman IgE monoclonal antibodies, 7.12 and 4.15 (provided by Dr. A. Saxon). The remainder of the plate was coated with one of the peanut or soy products at a concentration of 1 μg/ml in coating buffer (0.1 mol/L sodium carbonate-bicarbonate buffer, pH 9.5). The plate was incubated at 37° C for 1 hour and was 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. The upper two rows used a secondary standard reference to generate a curve for IgE, ranging from 0.05 to 25 ng/ml.

The serum pool (peanut positive or soy positive) was diluted (1:20 vol/vol) with various concentrations of the inhibiting product (0.00005 to 50 ng/ml) and incubated for 18 hours. The inhibited pooled serum (100 μl) was then dispensed in duplicate in the lower portion of the plate. After incubation for 1 hour at 37° C and washing, biotinylated, affinity-purified goat antihuman IgE (KPL, Gaithersburg, Md.) (1:1000 vol/vol phosphate-buffered saline) was added to all wells. Plates were incubated again for 1 hour at 37° C, washed, and 100 μl horseradish peroxidase-avidin conjugate (Vector Laboratories, Burlingame, Calif.) was added for 30 minutes. After washing, the plates were developed by the addition of a buffer containing 0-phenylenediamine (Sigma). The reaction was stopped by the addition of 100 μl 2N-hydrochloric acid to each well, and absorbance was read at 492 nm (Titertek Multiscan; Flow Laboratories, McLean, Va.). The standard curve was plotted on log-logit paper by means of simple linear regression, and values for the pooled serum were read from the curve as "nanogram-equivalent units" per milliliter (nanogram per milliliter).

An alkaline phosphatase competitive ELISA-inhibition analysis was done to compare IgG antifood specific antibodies from an assay previously published.[18, 19] The upper two rows of a 96-well microtiter plate (Gibco) were coated with 100 μl of a monoclonal antibody to human IgG (Miles Scientific; Naperville, Ill.). The remainder of the plate was coated (100 μl/well) with one of the peanut or soy products at a standard concentration of 5 μg/ml in coating buffer. The plate was incubated at 37° C for 1 hour and then washed five times with rinse buffer immediately and between subsequent incubations. The upper two rows used a standard reference to generate a curve for IgG, ranging from 5 to 250 μg/ml. The pooled serum samples were diluted with the inhibitor as described above and dispensed (100 μl/well) in the lower portion of the plate. After incubation for 1 hour at 37° C and washing, alkaline phosphatase conjugated goat antihuman IgG (Tago) was added to all wells. After incubation for 1 hour at 37° C and washing, nitrophenyl phosphate in carbonate buffer was added and the plate was developed. Plates were then read at 405 nm (Titertek Multiscan). The standard curve was plotted on log-logit paper, and values were read from the curve in "nanogram-equivalent units" per milliliter (nanogram per milliliter).

The percent inhibition was calculated by taking the food-specific IgE (or IgG) value minus the incubated food-specific IgE (or IgG) value divided by the food-specific IgE (or IgG) value. This number is multiplied by 100 to get the percentage of inhibition. The graphs from the above data plot percent inhibition versus log concentration of the inhibitor.

Chemical denaturation experiments

Chemical and enzymatic denaturation of crude and purified allergen components attempted to mimic human digestion. The immobilized digestive enzyme assay (IDEA) system was used for this purpose.[20, 21] This system consists of two bioreactors; one that stimulates gastric conditions and the other, conditions of hydrolysis in the small intestine. The use of immobilized proteinases and peptidases conserves the enzymes and prevents autolysis so that the same bioreactor can be used for numerous assays. Crystalline

porcine pepsin, trypsin, and chymotrypsin and crude intestinal mucosa peptidases (Sigma) were used for the bioreactors. The crude intestinal peptidases were further purified by ion-exchange chromatography with use of a Zetaprep QAE disk (AMF Corp., Merriden, Conn.). Controlled-pore glass (80 to 120 mesh CPG-2000, Electronucleonics, Fairfield, N.J.) was acid-cleaned and siliconized to form aminopropyl-glass as previously described.[21] Succinamidopropyl-glass was prepared by treating the aminopropyl-glass with a solution of 10% triethylamine and 5 gm succinic anhydride per gm of glass in acetone. Trypsin and chymotrypsin were immobilized on succinamidopropyl-glass by recirculation of a solution of the enzymes at pH 7.0 containing 10 mmol/L 1-ethyl-3-[3-(dimethylamino)propyl] carboimiide (EDC) through a column of the beads. Intestinal peptidases were immobilized on aminopropyl-glass by recirculation of a solution of the enzyme at pH 7.0 adjusted to 10 mmol/L EDC through a column of the beads at 4° C. Pepsin was immobilized by use of a sequential activation/immobilization procedure.[22] Approximately 5 gm succinamidopropyl-glass beads were activated with 50 ml 0.01 mol/L EDC in 0.1 mol/L NaCl, pH 7, for 20 minutes at room temperature. After activation, the beads were rapidly washed with ice cold 0.1 mol/L NaCl at pH 7, and immediately, 60 ml of a 10 mg/ml solution of pepsin was recirculated through a column of the beads for 2 hours at room temperature.

The pepsin bioreactor contained 1.5 ml of immobilized pepsin (bead specific volume, 3.4 ml/gm). Before use, each reactor was equilibrated with sample buffer. The sample was digested by recirculation of 15 ml through a fluidized-bed of the beads for 18 hours at 37° C. The pepsin-treated sample was collected and adjusted to pH 7.5 by addition of solid sodium phosphate. The "intestinal" bioreactor contained 0.2 ml immobilized trypsin, 0.3 ml chymotrypsin, and 1.0 ml of intestinal peptidases separated by porous polyethylene disks. The bioreactor was washed with 10 ml of the pepsin hydrolysate and the washing discarded. The remaining sample (5 ml) was recirculated through the bioreactor for 25 hours at 37° C. All proteins were subjected to both the pepsin and "intestinal" bioreactor.

Statistical analysis

ELISA-inhibition results were evaluated by the parallel-line assay developed by the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration.[23] All assays met four criteria: (1) minimum of four data points on the inhibition curve, (2) data points bracketed the 50% inhibition point, (3) linear correlation coefficient of 0.90 or greater for each inhibition curve, and (4) slopes of inhibition curves and standard curve were not statistically different by Student's paired t test at $p < 0.01$.

RESULTS

ELISA

Fig. 1 shows the results of the IgE- and IgG-specific ELISA-inhibition analyses to crude soy, soy whey, 7S, and 11S proteins with crude soy as the solid phase. By statistical analysis all inhibition curves were parallel.[23] Heat treatment at different temperatures for varying lengths of time did not change the IgE- or IgG-specific binding to any of the proteins. The inhibition curves were very similar without significant changes in the percent inhibition.

Fig. 2 shows the results of the IgE- and IgG-specific ELISA-inhibition analyses to crude peanut, 63.5 kd peanut allergen, and 17 kd peanut allergen with crude peanut as the solid phase. As was seen with the soy allergens, there was no significant inhibition in either the IgE- or IgG-specific binding to the allergens. By statistical analysis all inhibition curves were parallel except for two.[23] There was minimal change in inhibition in the 63.5 kd IgG-ELISA but not in the IgE-ELISA-inhibition analyses, when the protein was heated at 100° C for 20 minutes or 60 minutes, but the statistical comparisons were not valid because these two inhibition curves were not parallel to the other curves.

We used the IDEA system to determine if the digestive process would alter IgE-binding in an ELISA-inhibition analysis with either crude soybean or peanut as the solid-phase material. This system allows a protein to be passed over the columns to compare predigestion and postdigestion fractions. By statistical analysis all inhibition curves were parallel.[23] For soy protein there was only a 10-fold difference in the amount required to produce 50% inhibition (Fig. 3). When the crude peanut protein was digested in a similar manner the amount of protein required to produce 50% inhibition was approximately 100-fold greater for the digested protein.

DISCUSSION

Most food allergens appear to be naturally occurring food proteins. Foods contain an enormous variety of proteins but very little is known about the characteristics of most of these proteins.[24] The immunogenicity of a particular protein is likely related to its degree of "foreigness" to the host.[25] A complex protein with numerous antigenic determinants is more likely to be a better immunogen than a smaller protein with fewer antigenic determinants.[26] No unique features have been found to distinguish food allergens as a subset of antigenic molecules.[13] It appears that certain individuals are likely to be genetically more prone to develop significant IgE responses than others and thus have atopic disease. Several factors play a prominent role in the development of specific atopic disease. The patient's genetic makeup and other host related factors as well as the route of allergen administration, the dosage, and the frequency of exposure are important in this process to produce clinically apparent IgE-mediated disease. In children with positive

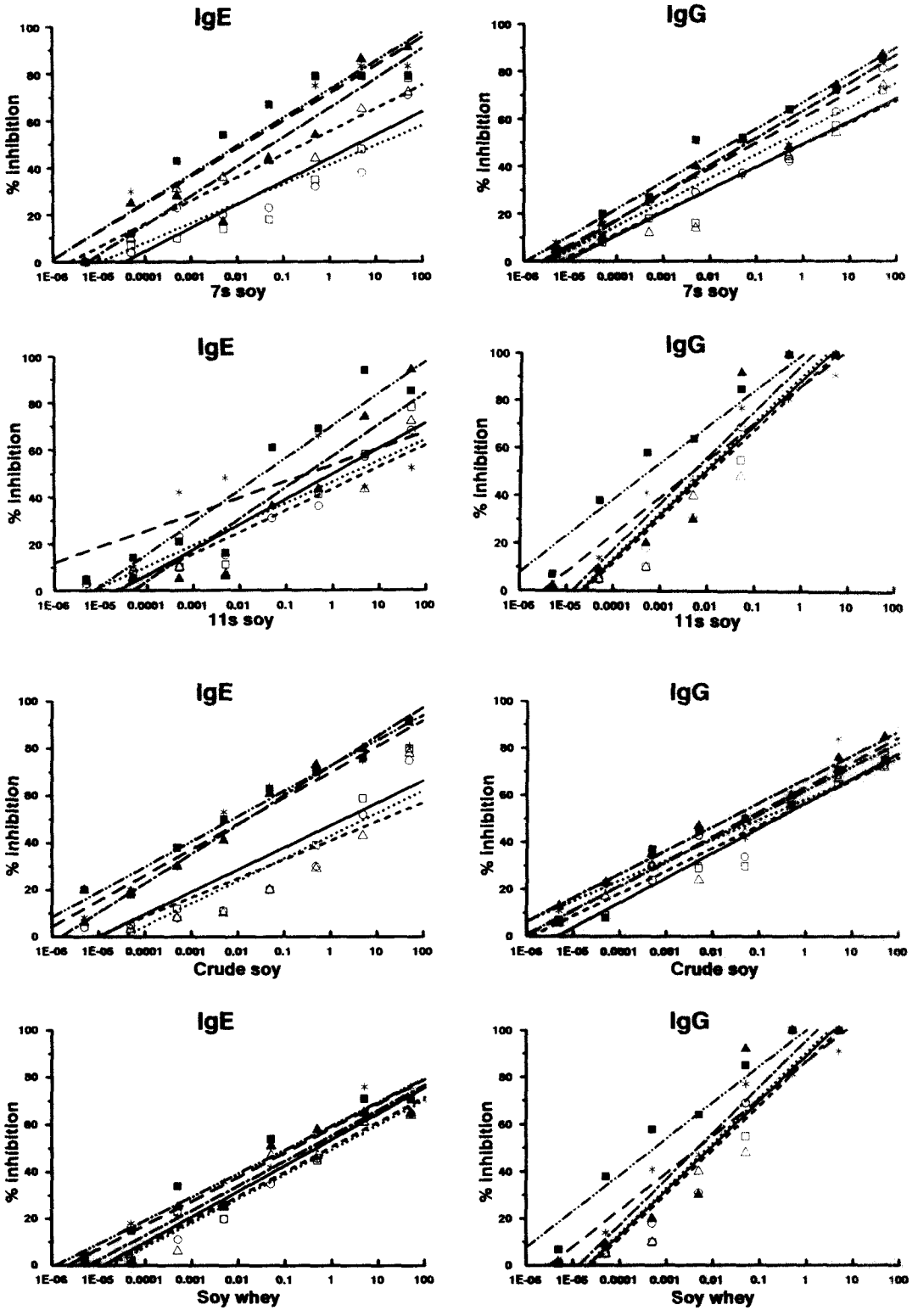

FIG. 1. IgE- and IgG-specific ELISA-inhibition analysis with crude soybean protein, soybean whey, 7S soybean fraction, and 11S soybean fractions. The untreated protein in each study was used as the solid-phase material. The soybean products were subjected to the following treatments: room temperature for 1 hour (□ - - - □), 37° C for 1 hour (△ . . . △), 56° C for 1 hour (○——○), 100° C for 5 minutes (*——*), 100° C for 20 minutes (■ ..-..- ■), and 100° C for 60 minutes (▲ .—.-—▲). Graphs plot percent inhibition versus log concentration of the inhibitor.

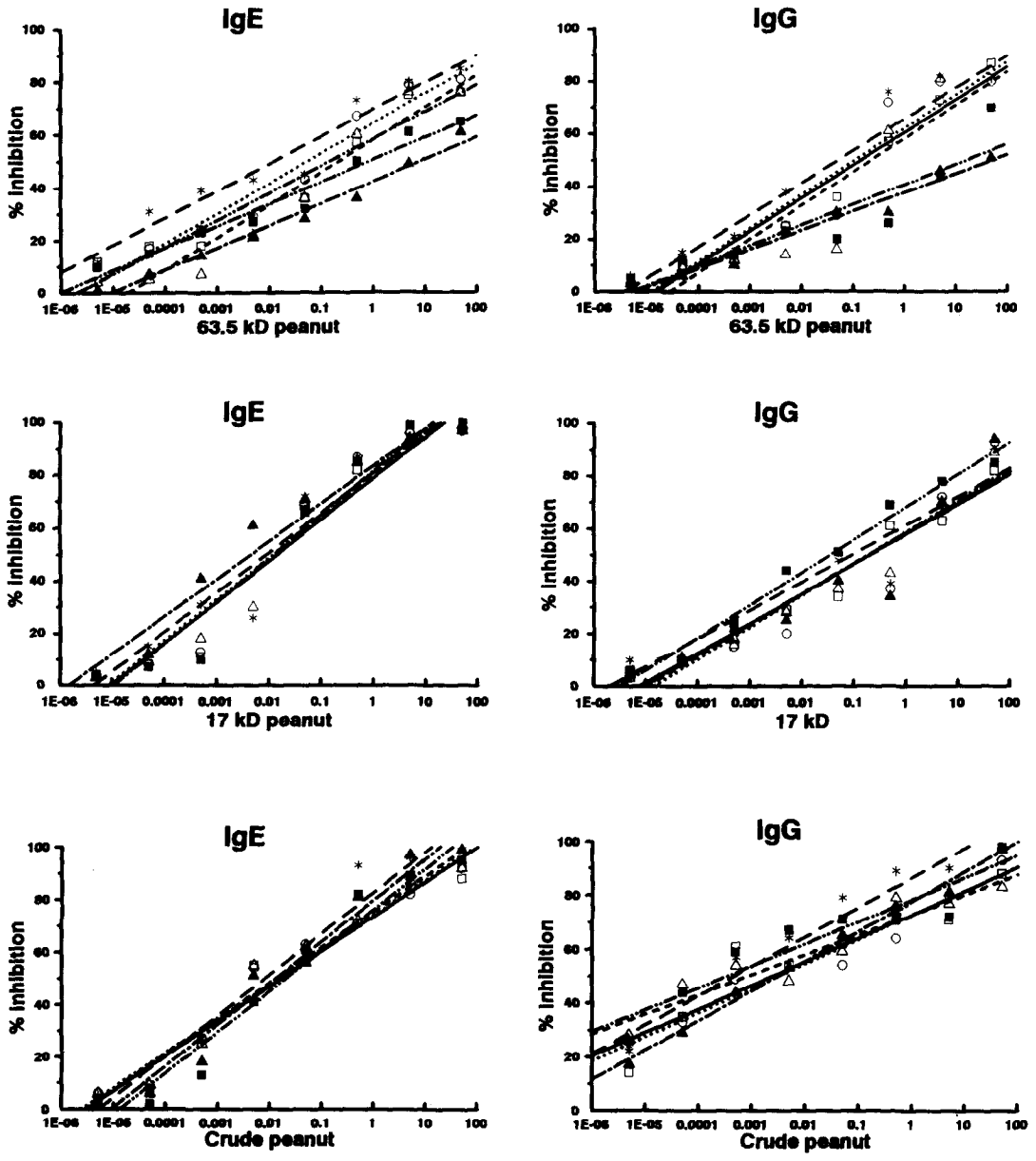

FIG. 2. IgE- and IgG-specific ELISA-inhibition analysis with crude peanut protein, 63.5 kd peanut allergen and 17 kd peanut allergen. The untreated protein in each study was used as the solid-phase material. The proteins were subjected to similar treatments as the soybean proteins. Graphs plot percent inhibition versus log concentration of the inhibitor.

DBPCFCs, six foods account for 90% of the positive food challenges.[8-10] This concentration of sensitivity to a small number of foods suggests that some characteristics of the antigen/allergen must be important in causing food hypersensitivity reactions.

Numerous articles have documented adverse food reactions to peanut and soy products.[27] A few studies have been completed to examine the allergenic fractions of these two proteins.[14, 28-30] Approximately 90% of the seed proteins in soybeans are salt-soluble globulins, with the bulk of the remainder being water-soluble albumins.[31, 32] These proteins comprise a broad range of molecular sizes and display four major sedimentation components, designated 2S, 7S, 11S, and 15S.[33] Each fraction is a complex mixture of proteins. The 7S and 11S each make up approximately one third of the extractable proteins followed by the 2S and 15S, which comprise 20% and 10%, respectively.[34] Peanut proteins are also divided into albumins and globulins.[27] The globulins are separated in two fractions known as arachin and conarachin.[27] In our previous studies we have purified by anion exchange

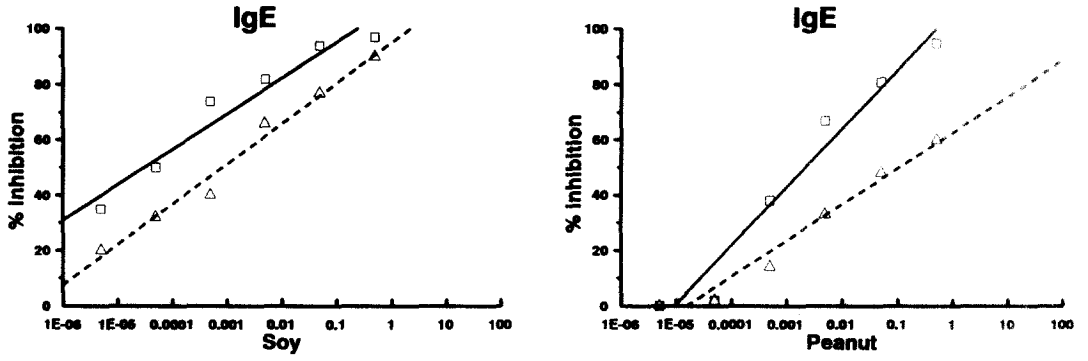

FIG. 3. IgE-specific ELISA-inhibition assay with crude peanut and crude soybean as solid-phase material, comparing predigestion and postdigestion fractions in the IDEA system as the inhibitors. Predigestion (□——□), postdigestion (△ . . . △). Graphs plot percent inhibition versus log concentration of the inhibitor.

chromatography two separate allergens that bind IgE from patients who had positive DBPCFCs to peanut. We identified a 63.5 kd allergen that had an isoelectric point (pI) of 4.55 (*Ara h* I).[6] The second allergen identified had a molecular weight of 17 kd and a pI of 5.2 (*Ara h* II).[15]

A previous study by Shibasaki et al.[28] examined the allergens in soybeans and the change in IgE binding after heat treatment. In their RAST and RAST-inhibition studies the 2S globulin had the highest IgE binding. In addition, the proteins were heated to 80° C, 100° C, and 120° C for 30 minutes. The activity of the 2S fraction was enhanced when heated to 80° C, but overall the IgE activity of the other fractions was reduced 40% to 75% of the untreated protein. Heiner et al.[35] examined the allergenicity of peanut components by RAST. After roasting it appeared that certain fractions had increased IgE binding, whereas others had significantly reduced IgE binding.

A few studies have examined the change in allergenicity of other food proteins. Heating of the milk proteins will denature bovine serum albumin, bovine gamma-globulin, and alpha-lactalbumin.[3] Less affected by heating are the milk proteins alpha-casein and beta-lactoglobulin.[3] In theory, alteration of the food protein may enhance the allergenicity of any one fraction. Mild heating of milk in the presence of lactose is known as the "browning reaction."[3] It is speculated that this reaction can increase the allergenicity of beta-lactoglobulin.[36] How enzymatic digestion of food proteins would effect the allergenicity of a particular protein has received much debate. New antigenic determinants were apparently demonstrated after the in vitro exposure of milk to the proteolytic enzymes pepsin and trypsin.[37] Another study with milk proteins showed reduced allergenicity by RAST to pepsin digests of beta-lactoglobulin.[38] *Gad c* I, the major allergen in codfish, in which sequential antigenic sites were identified, did not show any decrease in IgE binding after being subjected to reduction and alkylation.[39, 40] In a recent study, the effect of heating on tuna and salmon allergens was studied in a patient population with documented fish hypersensitivity.[41] They found by immunoblot analysis that canned tuna extracts revealed a striking loss of definable protein bands when compared with raw and cooked tuna extract.

Similar questions about the alteration of the allergenicity by chemical or thermal denaturation have been examined in other non-food-related allergens. Recently Lombardero et al.[11] used murine monoclonal antibodies and human IgE (from mite allergic patients) to determine the conformational stability of B-cell epitopes on the group I and group II mite allergens. The binding of group I allergens by monoclonal antibodies or human IgE was reduced 10- to 1000-fold after heating for 1 hour at more than 75° C, treatment at pH 2.0 or 12.0, or treatment with 6 mol/L guanidine or 6 mol/L urea. In contrast, the group II allergens were heat stable, resistant to pH changes, and resistant to denaturation with 6 mol/L guanidine. Both allergens are potent immunogens in human beings.

Similar studies to examine the effect of thermal or chemical denaturation with *Amb a* I, the major ragweed allergen, have shown the molecule, after treatment, to be virtually nonreactive with human IgG and IgE antibodies and monoclonal antibodies raised against *Amb a* I.[12, 26, 42] In addition, *Lol p* I, the major rye grass allergen is resistant to reduction and alkylation changes in IgE binding.[13, 43]

The debate has continued for some time to ascertain whether chemical or thermal treatment would enhance or reduce IgE binding to food proteins. Conflicting evidence based on the food or allergen source has been

published during the last several years. From our data in patients with documented positive DBPCFCs to peanuts or soybeans, chemical treatment designed to mimic human digestion or heat treatment to denature crude proteins and the allergenic fractions does not either enhance or significantly reduce IgE or IgG binding. These particular allergens appear to be stable to the degradation procedures presently investigated. Future oral challenge studies with similarly treated proteins would help to delineate the clinical relevance of this point.

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