

Immune Reactivities to Peanut Proteins, Agglutinins, and Oleosins

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ABSTRACT

Context • Certain individuals are sensitive enough to react to peanuts and peanut oil, sometimes with deadly effect. It is thus crucial to have an accurate testing methodology for the assessment of allergies and immune reactivities to peanuts and their components, such as agglutinins and oleosins. Currently, skin-prick testing is performed only with the water-soluble components of peanut proteins and can produce false negatives. Testing with all possible food antigens and with both immunoglobulin G (IgG) and immunoglobulin E (IgE) antibodies may offer a more accurate alternative.

Objective • The research team intended to measure IgG and IgE antibodies against peanut proteins, agglutinins, and oleosins to identify variations in IgG and IgE immune reactivities to these antigens among the general population.

Design • Sera from 288 healthy individuals—144 males of different ethnicities, aged 18–65 y with a median age of 35.5 y, and 144 females of different ethnicities, aged 18–65 y with a median age of 36.2 y—were obtained from Innovative Research, Inc. Four sera from patients with a known allergy to peanuts and 4 sera from individuals with no known allergy to peanuts were used as positive and negative controls. Several wells in the microtiter plate were coated with unrelated proteins, such as human serum albumin, rabbit serum albumin, and bovine serum albumin and used only for the determination of any background in the enzyme-linked immunosorbent assay (ELISA).

Setting: Immunosciences Lab, Inc, Los Angeles, CA, USA.

Outcome Measures • The sera were screened for peanut-specific IgG and IgE antibodies against water-soluble proteins of peanut, peanut agglutinins, and peanut oleosins, using the ELISA. Color development was measured at 405 nM. For demonstration of the specificity of the antibodies, inhibition ELISA was performed with 4 sera that had very high levels of IgG and IgE antibodies.

Results • Using mean values as the cutoff, 19%, 17%, and 22% of the specimens tested for IgG antibodies and 14%, 11%, and 14% of the specimens tested for IgE antibodies produced high levels of antibodies against peanut proteins, agglutinins, and oleosins, respectively.

Conclusions • The study's findings support the proposition that IgE sensitization to foods may not necessarily coincide with positive prick tests to commercial extracts. Falsely negative skin testing or IgG, IgA, or IgE antibody testing is often linked to the nature of the preparation of the food antigens and their use in in-vivo and in-vitro testing. The study's results support the need to improve the quality of food extracts used in the diagnosis of allergies and immune reactivity to nuts and seeds. Testing should use all possible food antigens and measure both IgG and IgE antibodies. (*Altern Ther Health Med.* 2015;21(suppl 1):73-79.)

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Peanuts are among the most popular of many types of plant seeds that are consumed worldwide as snacks, toppings, garnishes, flavorings, and ingredients. Peanuts can be found in movie-house concessions, on cakes, and in candy and ice cream. They are also a source of highly useful plant oil. Unfortunately, this widespread use means that peanuts will inevitably fall into the hands of the small percentage of the population who are sensitive enough to react to them, sometimes with deadly effect. It is thus crucial, even vital, to have an accurate testing methodology for the assessment of allergies and immune reactivities to the peanut

and its allergenic components, such as proteins, oleosins, and agglutinins.

The energy in plant tissues is preserved in the form of proteins, carbohydrates, and lipids. These substances are stored in their own, small, subcellular particles that are called *protein bodies*, *starch granules*, and *oil bodies*, respectively.¹

Oil bodies are also called *lipid bodies* or *oleosomes*.¹ They are stably packed in aqueous environments (ie, the cystolic compartment of seed cells). Seeds and nuts are widely used in the food industry, mostly because they contain 20% to 30% proteins as well as 30% to 50% oils in the form of oleosomes. These oil bodies are composed of a core of oil molecules or triacylglycerol (TAG), surrounded by a phospholipid monolayer containing densely packed proteins called *oleosins*. These oleosins are relatively small proteins of 15 to 25 kDa.¹

Oleosins play a key role in the stability of seed-oil bodies through electronegative repulsion and steric hindrance (ie, the prevention or retardation of inter- or intramolecular interactions as a result of the spatial structure of a molecule). This structural function prevents freeze- or thaw-induced damage and the calescence (increased temperature) of oleosomes during seed desiccation.² Further, oleosins are suggested to be bifunctional enzymes that have both monoacylglycerol-acyltransferase and phospholipase activities,³ which play a role in seed maturation.

The oleosins are composed of different domains; the hydrophobic domain anchors the oleosins to the TAG core, whereas the hydrophilic termini reside on the surface.^{1,4} Vegetable cooking oils commonly extracted from various oily seeds are made out of the TAG molecules that tend to separate from the aqueous solution and form a transparent layer on the surface of the liquid phase.⁵

An agglutinin is a substance that causes particles to coagulate to form a thickened mass. Agglutinins act like antibodies that cause antigen aggregation. They can also be substances other than antibodies, such as sugar-binding protein lectins. Lectins are carbohydrate-binding proteins first discovered in plants and now generally known to be present throughout nature. It is estimated that approximately 30% of our foods contain lectins, some of which may enter our circulation because of their resistance to digestive enzymes.⁶ Incompletely digested lectins may affect our health.⁷⁻⁹

Food immune reactivity involves mainly the protein components of foods or food antigens. Even minute quantities of these proteins, when ingested by sensitive individuals, are capable of provoking severe reactions. Vegetable oils produced from a diverse range of plant species contain proteins that can be highly allergenic. Production involves the pressing of plant seeds, followed by a series of purification steps to refine the oils to the desired degree.^{10,11} Although this refining process results in an almost complete removal of proteins, sometimes enough proteins remain in an oil to provoke a reaction in a sensitive individual.¹² Therefore, ascertaining the protein content of various oils is important for the following reasons:

Risk Assessment. The identity of the protein component that is responsible for the potentially allergenic properties of a food and the amount of that component present in an oil are crucial for risk assessment.

Safety of Oil Products. Information about the protein content of oils, detected by refined methodologies, can help to establish thresholds of immune reactivities and, therefore, can contribute to the safety of the oil products.

Currently, not much data are available regarding the safe levels of allergens in oils such as soy, maize, sunflower, sesame, and palm oils. Peanut oil, however, has been most thoroughly studied as a major allergen, and in its unrefined form, it can provoke severe reactions in some individuals. For example, when allergic individuals were exposed to 10 to 50 mg of peanut flour, an allergic response was not observed. But when these highly sensitive individuals were exposed to 100 mg of peanut flour, a significant allergic response was observed.¹² Because oleosins in the oleosomes make up approximately 10% of the total seed protein, the threshold of safety for these proteins is only approximately 1.4 mg.

Refined vegetable oils are used in a wide variety of food products. The use of these oils may change from time to time and from product to product. However, several oils derived from plants, such as peanuts, soy beans, and sesame seeds, have been recognized as potent antigens and allergens. As a result, a serious ongoing discussion is occurring about labeling each oil individually based on its allergenic risk.¹²

Due to their protein structure and strong antigenicity, oleosins potentially are hidden allergens in refined oils and oil-derived products, capable of eliciting immunoglobulin E (IgE) and non-IgE-mediated immune reactivity.¹³⁻¹⁶ In fact, sera from patients who suffer from allergies to seeds and nuts have reacted with oleosin proteins from different nuts and seeds in different oils.^{16,17} Because oleosins are hidden and their epitopes are buried in the inner molecules, the preparation of most commercial nut and seed extracts actually does not remove the oleosins from the oils.

However, because all oleosins are fat soluble, not water soluble, an allergy testing method that uses nut and seed extracts prepared in aqueous solution would be using a preparation that actually does not contain oleosins.¹⁶ Therefore, for testing immune reactivities to oleosins, they must either be synthesized or purified through sophisticated biochemical steps.

Peanut oleosins have been studied extensively compared with other oleosins. For this reason, the current study measured immunoglobulin G (IgG) and IgE antibodies against peanut proteins, agglutinins, and oleosins to demonstrate variations in IgG and IgE immune reactivities to these antigens among the general population.

METHODS

Sera from 288 healthy individuals who were qualified to donate blood—144 males of different ethnicities, aged 18 to 65 years with a median age of 35.5 years, and 144 females of different ethnicities, aged 18 to 65 years with a median age of

36.2 years—were obtained from Innovative Research, Inc (Southfield, MI, USA).

Preparation of Peanut Antigens

Peanut agglutinin was purchased from Sigma Chemicals (St Louis, MO, USA). Peanut antigens were prepared from products purchased from the supermarket in both raw and roasted form. For that preparation, 10 g of mixed raw and roasted peanuts were put in a food processor using 0.1 M of phosphate buffer saline (PBS) at pH 7.4. The mixer was turned on and off for 1 hour and then kept on the stirrer overnight at 4°C. After centrifugation at $20\,000 \times g$ for 15 minutes, the top layer, which contained oil bodies, was discarded. The liquid phase was removed and dialyzed against 0.01 M of PBS using dialysis bags, with a cutoff of 6000 kDa. Dialysis was repeated 3 times to make sure that all small molecules were removed. After dialysis, protein concentrations were measured using a kit provided by Bio-Rad (Hercules, CA, USA).

Purification of Peanut Oleosin

To purify the oleosin from the peanuts, the peanuts were prepared according to the method described above and elsewhere.¹³ A total of 100 mL of chloroform/methanol (2/1, v/v) was then added and blended for 2 minutes using a food processor. The mixture was put in a 50-mL tube and centrifuged at 14 000 RPM for 5 minutes. The liquid in the upper phase was filtered through 2 layers of filter paper. The resultant filtrate was collected in multiple glass bottles and dried under a stream of air, with strong continuous agitation. The chloroform/methanol extraction step was repeated twice.

A total of 20 mL of diethyl ether was then added, and the white, solid material stuck on the surface of the glass bottles was detached and resuspended in diethyl ether. At this point, 10 mL of water was added to each bottle, which was centrifuged at $20\,000 \times g$ for 5 minutes. The upper diethyl ether layer that contained lipids was removed, and the white, solid, interface material containing the oleosins was collected and transferred to microtubes with a minimum volume of water and diethyl ether.

The microtubes were centrifuged at $20\,000 \times g$ for 5 minutes. The interfacial material was exposed to a stream of nitrogen to evaporate the remaining diethyl ether. One mL of chloroform/ethanol (95/5, v/v) was added to the interfacial material in each tube. The contents of each tube were quickly vortexed and transferred to a glass flask.

To separate any protein contaminants from the oleosins, 10 mL of chloroform/methanol (95/5, v/v) was added, and the mixture was filtered through filter paper that was previously rinsed with chloroform/methanol. The filtrate was collected in a flask and dried under a stream of nitrogen. The dried oleosins were dissolved in chloroform/methanol and applied to a Sephadex LH-60 column (Bio-Rad, Hercules, CA) using chloroform/methanol as the solvent. The collected fractions of oleosins were checked by sodium dodecyl sulfate (SDS)-gel electrophoresis.¹⁸

Outcome Measures

Enzyme-linked Immunosorbent Assay for IgG and IgE Measurement. For the Enzyme-linked Immunosorbent Assay (ELISA), peanut proteins, agglutinin, and oleosin at a concentration of 1 mg/mL were dissolved in 0.1 M of PBS. Next, these antigens were diluted 1:50 in 0.1 M of carbonate buffer at pH 9.2, and 100 mL or 2 mg of each antigen was added to wells of a microtiter plate and incubated overnight at 4°C. After washing, the unoccupied sites in the wells were saturated by adding 200 mL of 2% bovine serum albumin (BSA), with these wells functioning as controls, and were incubated overnight at 4°C. The plates were washed again, and 100 mL of different sera was diluted at 1:4 for IgE- and 1:100 for IgG-level determination, and the diluted sera were then added to duplicate wells of each microtiter plate and incubated for 1 hour at room temperature.

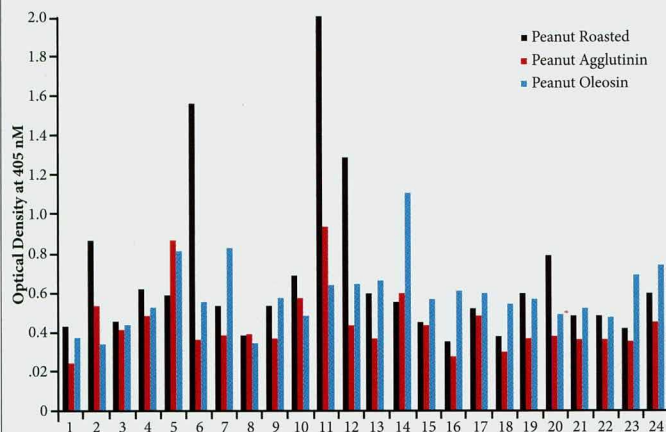
This procedure was followed by washing and the addition of optimal dilutions of alkaline-phosphatase-labeled antihuman IgE to 1 set and antihuman IgG to a different set of plates, followed by incubation for 1 hour at room temperature. After another washing and the addition of a substrate to the wells, para-nitrophenyl phosphate, the color development was measured at 405 nM. Four sera from patients with a known allergy to peanuts and 4 sera from individuals with no known allergy to peanuts were used as positive and negative controls. Several wells were coated with unrelated proteins, such as human serum albumin (HSA), rabbit serum albumin (RSA), and BSA and used only for the determination of any background in the ELISA.

ELISA Inhibition. Inhibition ELISA was performed for demonstration of the specificity of the antibodies. Four sera with very high levels of IgG and IgE antibodies against roasted peanut protein, peanut agglutinins, and peanut oleosins were prediluted 1:4 for IgE and 1:100 for IgG determination, and they were preincubated with 100 mg of HSA, RSA, and peanut-specific antigens for 2 hours at 37°C and 2 hours at 24°C. After centrifugation at $3000 \times g$, 100 mL of each supernatant was transferred to a microtiter plate coated with peanut protein, peanut agglutinin, and peanut oleosin. After 4 hours of incubation at 24°C, the ELISA procedure was continued according to the standard procedure. Results were expressed as a percentage inhibition after subtraction of the optical density (OD) of the background (ie, the wells coated with HAS).

RESULTS

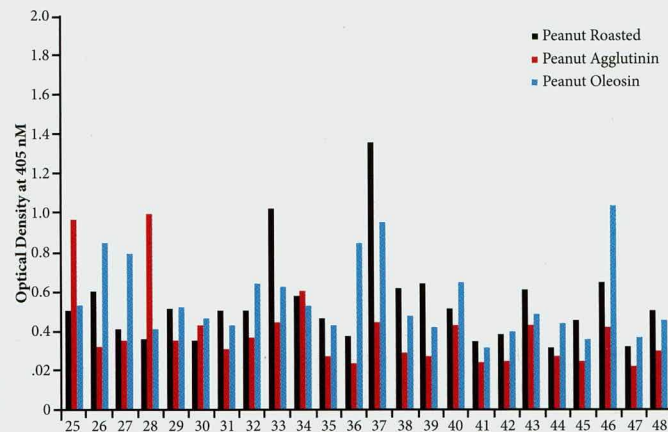
Sera from 288 individuals were screened for peanut-specific IgG and IgE antibodies against water-soluble proteins of peanut, peanut agglutinin, and peanut oleosin. For the 3 antigens, Figures 1 through 4 summarize the data for IgG antibodies for 98 of the 288 tested specimens. The cutoffs for all testing were at the mean ODs for all 288 specimens. At a cutoff of 0.62 OD for peanut proteins, 55 of the tested specimens (19%) had a significant elevation in those antibodies. At a cutoff of 0.45 OD for peanut agglutinins, 49 of the specimens (17%) showed an elevation in IgG

Figure 1. Optical density at 405 nM for specimen Nos. 1-24 tested for IgG antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



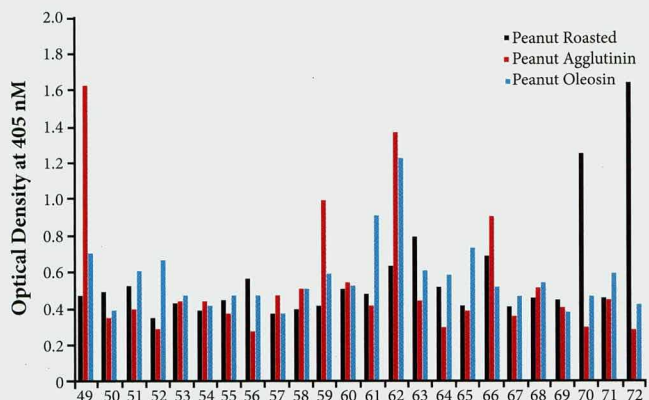
Abbreviation: IgG, immunoglobulin G.

Figure 2. Optical density at 405 nM for specimen Nos. 25-48 tested for IgG antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



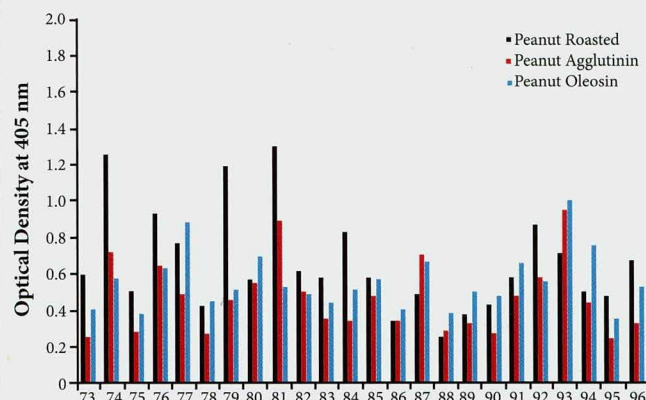
Abbreviation: IgG, immunoglobulin G.

Figure 3. Optical density at 405 nM for specimen Nos. 49-72 tested for IgG antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



Abbreviation: IgG, immunoglobulin G.

Figure 4. Optical density at 405 nM for specimen Nos. 73-96 tested for IgG antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



Abbreviation: IgG, immunoglobulin G.

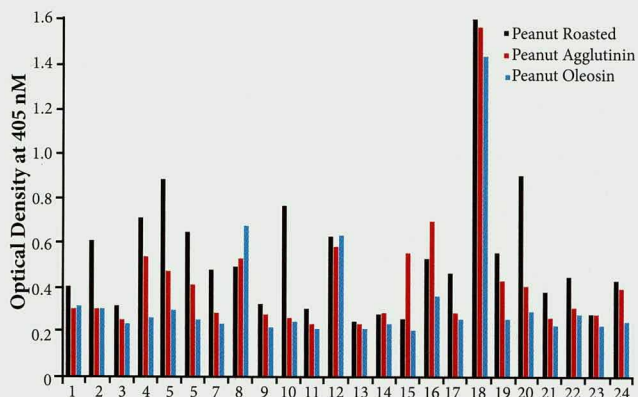
antibodies. For peanut oleosin, 63 of the specimens (22%) exhibited an elevation in IgG antibodies at a cutoff of 0.54 OD. Whereas some individuals reacted only against peanut proteins, and others reacted against only agglutinin or oleosin, only 3 specimens were reactive against combinations of 2 or of all 3 peanut allergens simultaneously.

Data related to IgE-specific antibodies against peanut proteins, agglutinins, and oleosins are presented in Figures 5 through 8. At a cutoff of 0.65 OD, 41 of the tested specimens (14%), showed an elevation in the levels of IgE antibodies against peanut proteins. Of this 14%, 4% had moderate

elevations, and the other 10% had very significant elevations in IgE antibodies. At a cutoff of 0.46 OD, 15 of the tested specimens (5%) had very significant elevations of IgE antibodies against peanut agglutinins, and an additional 18 (6%) showed moderate elevations. Finally, at a cutoff of 0.42, 16 (6%) exhibited very high levels of IgE antibodies for peanut oleosins, and an additional 24 (8%) showed moderate elevations.

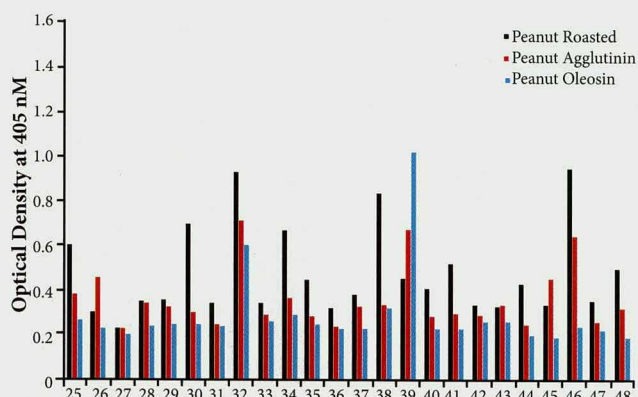
Further analysis of the data showed that almost 50% of the specimens showed only IgE-specific antibodies against water-soluble components of peanut proteins, whereas the

Figure 5. Optical density at 405 nM for specimen Nos. 1-24 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



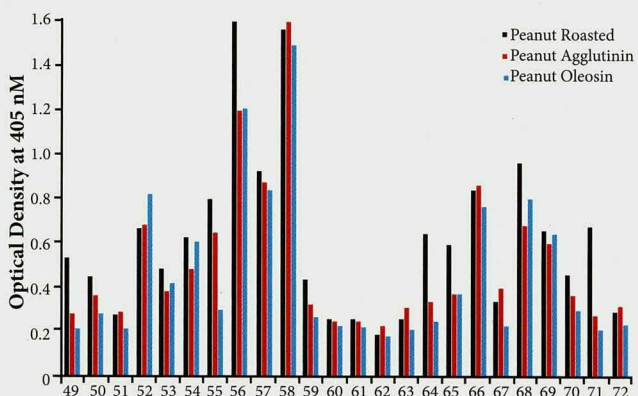
Abbreviation: IgE, immunoglobulin E.

Figure 6. Optical density at 405 nM for specimen Nos. 25-48 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



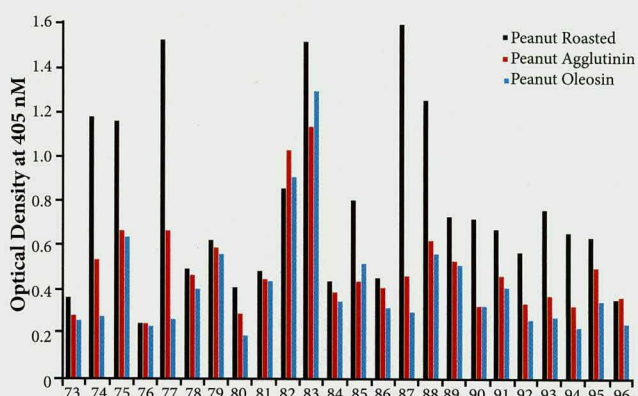
Abbreviation: IgE, immunoglobulin E.

Figure 7. Optical density at 405 nM for specimen Nos. 49-72 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.



Abbreviation: IgE, immunoglobulin E.

Figure 8. Optical density at 405 nM for specimen Nos. 73-96 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.

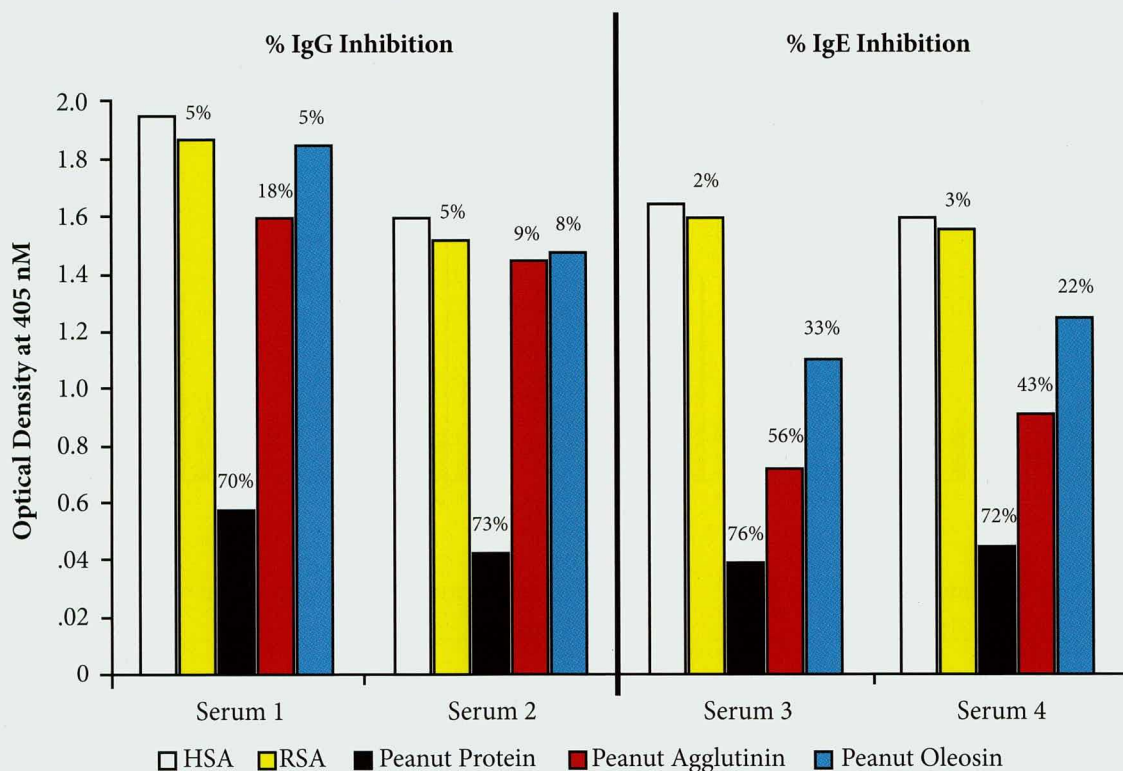


Abbreviation: IgE, immunoglobulin E.

other 50% showed simultaneous elevations in IgE antibodies against peanut proteins and peanut agglutinin; peanut proteins and peanut oleosin; or peanut proteins, peanut agglutinin, and peanut oleosin. In only 11 of the 288 tested specimens (4%) were the elevations for oleosin antibodies much greater than those for agglutinin or peanut proteins. To demonstrate the specificity of this immune reaction and possible cross-reactivity between peanut proteins, agglutinin, and oleosin, inhibition with the specific and nonspecific antigens was performed. Although the addition of roasted-peanut proteins to 2 different sera with high levels of IgG

antibodies against this protein resulted in a 70% to 73% inhibition of IgG reactivity with plates coated with peanut proteins, the addition of peanut agglutinin and oleosin resulted only in 8% to 20% inhibition of this assay (Figure 9). Although a similar inhibition with specific antigens was observed for inhibition of IgE antibodies, the addition of peanut agglutinin and oleosin resulted in more significant inhibitions of IgE antibodies. For example, the percentage inhibitions for agglutinin and oleosin for one serum were 56% and 33%, respectively, and for a second serum were 43% and 22%, respectively.

Figure 9. Determination of IgG- and IgE-specific antibodies against roasted peanut proteins in the presence of nonspecific antigens HSA, RSA, peanut roasted, peanut agglutinin, and peanut oleosin.



Abbreviations: IgG, immunoglobulin G; IgE, immunoglobulin E; HSA, human serum albumin; RSA, rabbit serum albumin.

DISCUSSION

Food immune reactivities represent an increasingly prevalent problem that has significant adverse effects on an individual's quality of life.¹⁹ Among more than 200 foods that cause immune reactivity, peanut proteins represent a particularly well-known and well-documented threat for sensitive individuals worldwide.²⁰ Peanuts and peanut butter are favorite foods in homes in the United States as well as in many other countries. Peanuts contain a high percentage of protein (25%-30%), oil (40%-50%), and a lot of valuable nutrients. Because of their high fat content, peanuts are used as an excellent source of oil.²⁰

So far, more than 32 peanut allergens that can bind to IgE and IgG have been identified. The majority of these allergens, including peanut agglutinin, are water-soluble glycoproteins and are stable to heat, stomach acid, and enzymatic digestion. However, the hydrophobic proteins called *oleosins* found in the membranes of oil bodies stored in nuts and seeds are not water soluble. Almost all commercially available peanut antigens are prepared from water-soluble proteins.¹⁵ This fact could be the reason why many cases of anaphylaxis have been reported despite negative results for both in vitro, IgE-specific test and in vivo, skin-prick tests using commercially available antigens.^{21,22}

Because of this issue, the current study put a major emphasis on measuring both IgG and IgE against 3 different groups of peanut antigens: water-soluble proteins, pure

peanut agglutinin, and peanut oleosin. First, the current author wanted to find the percentage of healthy individuals who produce such a significant level of IgE antibodies against these antigens that their exposure to them may indicate a risk of exhibiting type 1 allergic reaction. The data summarized in Figures 5 through 8 show that 14%, 11%, and 14% of 288 tested sera exhibited elevation of IgE antibodies against water-soluble proteins, agglutinin, and oleosin in peanuts, respectively. This finding indicates that both peanut oleosin and agglutinin, as peanut allergens, are equally as important as water-soluble peanut proteins and, therefore, in vitro or in vivo testing with water-soluble peanut proteins alone can result in many false-negative results.

Further, researchers have suggested that severe allergic reactions in the face of a lack of detection of IgE antibodies may mean that an anaphylaxis was IgG mediated.^{21,22} Other data have confirmed that the potent immunogenicity of oleosin induces a polyisotypic—meaning an IgG, an IgA, and, in some cases, an IgE response—supporting the assumption of anaphylactic reaction by non-IgE antibodies.²¹⁻²³ Precisely for that reason, the current study measured not only IgE but also IgG antibodies against all of the aforementioned peanut antigens. Results presented in Figures 1 through 8 showed that approximately 30% of the antibody-reactive individuals produced both IgG and IgE simultaneously. For the other 70%, some produced only IgG antibodies, whereas others produced only IgE antibodies.

ELISA inhibition proved that these antibodies were specific and that epitope similarity exists between peanut proteins, peanut agglutinin, and peanut oleosin to some extent regarding the production of IgG antibodies and, to a greater extent, of IgE antibodies.

In addition, in a study of 32 patients displaying immediate symptoms of allergic reaction, such as asthma, urticaria, and angioedema, sesame allergy was diagnosed by a convincing clinical history and double-blind, placebo-controlled food challenges. However, of the 32 patients, 10 had negative prick and IgE-specific test results using available saline-extracted antigens. When the oil-soluble component of sesame was prick-tested and the patients were tested against sesame oleosin, the sera from all patients reacted with this fraction of the sesame protein.¹⁶ The researchers concluded that oleosins are major allergens of sesame seeds and can be relevant to severe anaphylaxis. Falsely negative prick tests could be due to the lack of oleosin in presently available extracts or to the fact that the epitopes were buried in the inner molecule. Detection tests currently used to identify sesame allergens based on sesame vicillins or other storage proteins may be insufficient for the detection of sesame immune reactivities.¹⁶

The current study's findings support the proposition that IgE-sensitization to foods may not necessarily coincide with positive prick tests to commercial extracts, because a maximum of diagnostic sensitivity is difficult to achieve. However, some studies point to the possibility that false-negative skin testing or IgG-, IgA-, or IgE-antibody testing is often linked to the nature of the preparations of the food antigens.²⁴⁻²⁶ Those studies and the results presented for the current study support the obvious need to improve the quality of food extracts used in the diagnosis of allergies and immune reactivity to nuts and seeds, in which oleosins correspond to 1% to 2% of total seed and nut proteins. This fact means that a few milliliters of oil is enough to cause hidden food immune reactivity while masking the source.^{15,27} The fact that oleosins are major allergens supports the adoption of oleosins as new markers for testing of food immune reactivity, because otherwise many false-negative results may be reported, bringing many years of suffering that could have been avoided.

Because many oleosins cross-react with each other, and even with other allergens, individuals with immune reactivity to oleosins should be aware of this cross-reactivity and consider the possible elimination of such cross-reactive foods from their diets. For example, recently it was found that peanut oleosin cross-reacts with buckwheat.²⁸ Therefore, if patients react to peanut oleosin, both peanut and buckwheat should be removed from their diets.

CONCLUSIONS

The author concludes that a more accurate detection of allergies and immune reactivity in vitro and in vivo requires that testing be performed with all possible antigens of the suspected, specific foods and that both IgG and IgE antibodies be measured.

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