

Legumes, eggs, and milk

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Approximately 6% of the pediatric population less than 3 years of age (1), and 2% of the general population are affected by food hypersensitivities (2). Although hundreds of different foods are present in the Western diet, about 80% of allergic reactions in children are the result of hypersensitivity to legumes (including peanuts and soybeans), cow's milk, and chicken eggs. Most children (~80%) "outgrow" their hypersensitivity to milk and eggs by the end of the first decade. However, peanut allergy persists in about 80% of young children reacting to peanut in the first 2 years of life, and virtually all individuals reacting to peanut beyond the age of 5 years (3, 4). Why these particular foods account for the vast majority of reactions in children is not clear, although their early introduction into the diet appears to play some role.

Food items comprise proteins, carbohydrates, and lipids. In general, the major food allergens identified are water-soluble glycoproteins, which have molecular weights ranging from 10 to 70 kDa and are stable to processing and cooking (heating, freezing, etc.) and gastrointestinal digestion (gastric acid and intestinal proteases) (5). However, other physicochemical properties that may account for the unique allergenicity of specific foods are poorly understood; e.g., mucosal permeability, structural differences, and preferential uptake by different antigen-presenting cells (APCs). Major allergens identified in legumes, cow's milk, and egg are listed in Table 1. The surface portion of allergens that interact with T cells and B cells or antibodies are defined as T-cell and B-cell epitopes, respectively. T-cell epitopes comprise small peptides, about nine sequential amino acids, presented to T cells after processing by APCs and presented

in the context of the MHC complex (6). B-cell epitopes may be linear (sequential) or conformational (discontinuous), and generally comprise larger peptides (10–20 amino acids) than T-cell epitopes. Since food proteins undergo cooking and digestive processes, it generally has been assumed that most food allergenic epitopes (IgE-binding) are predominantly linear in nature. Recent work suggests that many of the food allergenic epitopes are conformational, and as discussed below, these structural differences in epitopes may produce different immunologic consequences.

Legumes

In the USA, peanuts and soybeans are among the most common food allergens in children, and peanuts among the most common in adults, although other members of the legume family also have been implicated in allergic reactions, i.e., green peas, green beans, navy beans, garbanzo beans, lupines, etc. Although extensive cross-reactivity among legumes is seen with *in vitro* methods (e.g., RAST, ELISA, and SDS-PAGE immunoblotting) and skin testing, most peanut-allergic patients can consume other legumes with no adverse reaction, and vice versa (7, 8).

Peanuts are one of the most allergenic foods in children and adults. Peanut proteins are conventionally classified as albumins (water-soluble) and globulins (saline-solution soluble), the latter of which is further subdivided into arachin and conarachin fractions (9). With crossed-radioimmuno-electrophoresis (CRIE), 16 allergenic fractions have been identified in raw peanuts (10). Thirty-two protein bands have been identified on SDS-PAGE; three with molecular weights of 63.5 kDa (Ara h 1) (11), 17

Table 1. Isolated food allergens

Protein fraction	Approx. percentage of food	MW
Legumes		
Peanut		
Ara h 1	—	63 500
Ara h 2	—	17 000
Ara h 3	—	14 000
Soybean		
Soybean trypsin inhibitor	—	20 500
Gly m 1	—	30 000
Cow's milk		
Caseins	76–86%	19 000–24 000
Whey	14–24%	
β-Lactoglobulin	7–12%	36 000
α-Lactalbumin	2–5%	14 440
Chicken egg white		
Gal d 1 (ovomucoid)	11%	28 000
Gal d 2 (ovalbumin)	54%	45 000
Gal d 3 (ovotransferrin)	12–13%	77 700
Gal d 4 (lysozyme)	3.5%	14 300

kDa (Ara h 2) (12), and 14 kDa (Ara h 3) (13, 14) have been identified as major allergens. Recently, we utilized soybean-adsorbed, peanut-allergic patient sera to identify IgE-binding peanut protein fractions on two-dimensional immunoblots. These fractions were isolated and then identified by N-terminal amino-acid sequencing. We showed that the ~30 protein fractions present represented isoforms or fractions of Ara h 1, Ara h 2, or Ara h 3 (15). A wide variety of peanut products, including flour and reprocessed peanuts, have been shown to retain their allergenicity (16). However, refined peanut oil was found to be safe in 60 peanut-allergic individuals, whereas pressed (or extruded) oils were found to retain some of their allergenicity (17).

Ara h 1 (64.5 kDa) belongs to the vicilin family of seed storage proteins. The entire amino-acid sequence has been determined, and a full-length cDNA clone encoding this allergen has been isolated and expressed (11, 18). With peanut-allergic patient sera and overlapping decapeptides representing the entire primary amino-acid sequence of Ara h 1, twenty-three different IgE-binding epitopes were identified, four of which were recognized by >80% of peanut-allergic patient sera. In further studies, mutational analysis of the immunodominant epitopes revealed that a single amino-acid substitution, especially in the center of each epitope, could dramatically reduce or abrogate IgE binding (19). Expression of these mutated recombinant proteins may prove valuable in the development of safer vaccines for immunotherapy.

Ara h 2 (17.5 kDa) is a member of the conglutin family of storage proteins, which has been fully sequenced and a full-length cDNA clone isolated (20). In addition, 10 IgE-binding epitopes have been identified, although three are clearly immunodominant, being recognized by sera from all patients tested: aa 27–36, aa 57–66, and aa 65–74. Mutational analysis of these major allergenic epitopes demonstrated that single amino-acid substitutions resulted in near complete loss of IgE binding. T-cell epitope mapping with peanut-specific T-cell lines demonstrated four immunodominant regions on the Ara h 2 molecule, three of which mapped to different locations than the immunodominant IgE-binding epitopes. The fact that IgE-binding epitopes differ from T-cell epitopes should enable us to mutate IgE-binding sites without affecting the response of T cells, a prerequisite for developing recombinant vaccines that will not induce anaphylactic side-effects.

Ara h 3 (14 kDa) is a member of the glycinin family of storage proteins (13, 14). Glycinins represent a class of seed storage proteins that are first synthesized as precursor proteins of ~60 kDa and then proteolytically cleaved into 40- and 20-kDa proteins. The 14-kDa protein identified by immunoblotting appears to represent a natural cleavage product of the amino terminal end of this peanut glycinin. A full-length cDNA of the 60-kDa protein has been isolated, and epitope mapping studies are underway. There does not appear to be any common amino-acid sequence motif shared by these three peanut proteins, although the hydrophobic amino-acid residues appear to play a critical role in each of the IgE-binding epitopes.

Soybeans are another member of the legume family that cause a significant number of hypersensitivity reactions, predominantly in infants and young children. Since soybeans provide an inexpensive source of high-quality protein, soybean protein is used in many commercial foods. Approximately 10% of the seed proteins are water-soluble albumins and the remainder are salt-soluble globulins (21). Four major protein fractions have been separated by ultracentrifugation: 2S (α-conglycinin composed of 18.2- and 32.6-kDa fractions), 7S (β-conglycinin exists as a trimer or hexamer in solution; monomeric form is 150–170 kDa), 11S (glycinin is 320–350-kDa protein composed of 12 subunits), and 15S (aggregated glycinin). In a study of eight children with IgE-mediated soy hypersensitivity documented by double-blind, placebo-controlled food challenge (DBPCFC), ELISA and immunoblots were used to compare IgE and IgG specific antibodies to the 7S, 11S, and whey fractions (21). In this small group, no fraction appeared to be more allergenic. An earlier study had suggested

that the 2S fraction had the highest allergenic potential (22), while a later study suggested that the 7S fraction contained the major allergenic component (23). Gly m 1 is a 30-kDa protein comprising a minor portion of the 7S conglycinin that was bound by IgE antibodies from 65% of soy-“allergic” patients in one study (23). Soy vicilin, highly homologous to peanut vicilin, has also been reported to be a major soy allergen. As with peanut oil, seven subjects reportedly allergic to soy were found to have no symptoms after ingesting up to 8 ml of soy oil (24). With ELISA inhibition, however, some detectable soy protein has been found in soy-fat products (25). Six of seven soy lecithin preparations contained 1.03–27.2 mg/g of soy protein, 4/5 margarines contained 0.1–0.29 mg/g, and 3/8 soy oil preparations contained 0.11–3.3 mg/g.

Cow's milk

Cow's milk is among the first foods introduced into an infant's diet and thus one of the most common causes of food allergy in young children (26). Prospective studies from several countries have shown that about 2.5% of neonates experience hypersensitivity reactions to cow's milk in the first year of life (1, 27, 29). IgE- and non-IgE-mediated hypersensitivity mechanisms each account for about one-half of milk-allergic disorders. While most infants with non-IgE-mediated cow's milk allergy “outgrow” their sensitivity by year 3 of life (27), 15% of infants with IgE-mediated cow's milk allergy retain their sensitivity into the second decade, and 35% develop allergic reactions to other foods (30).

Over 60 million tons of milk are produced in the USA each year; three-fourths is consumed as fluid milk or cheese (31). In addition, over 120000 metric tons of caseinates (sodium, potassium, and calcium) are produced for consumption annually worldwide. Caseinates are very soluble and extremely heat stable, and exhibit excellent emulsification, water-binding, thickening, and gelling characteristics. Consequently, they are used extensively throughout the food supply in formulated meat products, texturized vegetable protein products, margarines, toppings, cream substitutes, coffee whiteners, and foam stabilizers, making avoidance of milk proteins by milk-allergic patients extremely difficult.

Cow's milk proteins have been well characterized, but the relative allergenicity of each protein in various forms of milk hypersensitivity remains unclear. Cow's milk contains at least 20 protein components that may provoke an antibody response in man (32). The milk protein fractions traditionally have been subdivided into casein and whey fractions. The caseins are generally found in

micellar complexes, which give milk its “milky” appearance, and constitute 76–86% of the protein in cow's milk (33, 34). The casein fraction is precipitated from skim milk by acid at pH 4.6, and it consists of three basic caseins (α_s , β , and κ), which account for 80% of milk protein. The α_s caseins (α_{s1} and α_{s2}) constitute 42%, β casein 25%, and κ casein 9% of total milk proteins. The primary sequences of the caseins have been established (33), and five genetic variants of α_{s1} casein, four variants of α_{s2} casein, and two genetic variants of β casein have been identified. The α_{s1} caseins consist of 199 amino-acid residues with a calculated molecular weight of 23.6 kDa, and have no conformational structure since they lack disulfide bonds. Epitope-mapping by assays with overlapping peptides has demonstrated that major allergenic binding sites are in the C-terminal region of the α_s casein (35). The β caseins contain 209 amino-acid residues with a calculated molecular weight of 23.9 kDa, and the κ caseins are made up of 169 amino-acid residues with a calculated weight of approximately 19 kDa. The noncasein fraction, or whey, accounts for 20% of total milk protein. It consists of β -lactoglobulin (9% of total milk protein), α -lactalbumin (4%), bovine immunoglobulins (2%), bovine serum albumin (1%), and minute quantities of various proteins (lactoferrin, transferrin, lipase, esterases, etc. that collectively make up 4% of total milk protein). Several of the whey proteins are denatured by extensive heating (bovine serum albumin, bovine gamma globulin, and α -lactalbumin) (33). The complete primary structure of β -lactoglobulin has been elucidated, and allergenic linear epitopes have been mapped (36). β -Lactoglobulin is composed of 162 amino-acid residues with a calculated weight of 18.3 kDa. Similarly, α -lactalbumin has been shown to comprise 123 amino-acid residues with a calculated weight of 14.2 kDa.

Many authors have investigated the relative allergenicity and antigenicity of the milk protein but there has been little consensus. Goldman et al. skin tested 85 children who had challenge documented milk hypersensitivity, with a number of milk proteins (37). Fifty of the children had positive skin tests; 30/50 to two or more of the milk proteins: caseins – 68%; α -lactalbumin – 44%; β -lactoglobulin – 40%; and bovine serum albumin – 42%. Of 45 children challenged with “purified” milk proteins, 62% reacted to β -lactoglobulin, 60% to casein, 53% to α -lactalbumin, and 52% to bovine serum albumin (38). However, several factors raise significant doubts about the reported reactivity rates in this study: the use of the intradermal skin test method, the large number of investigators performing skin tests, and the difficulty of obtaining the

milk proteins in a pure form. Virtually all commercial sources of whey proteins are contaminated with casein proteins, and vice versa. Utilizing the RAST with purified milk proteins, Hoffman concluded that β -lactoglobulin was the major allergen in cow's milk (39). However, the diagnosis of milk allergy was based on history and skin test data; therefore, the true clinical sensitivity of these patients remains in doubt. Other studies employing CRIE have demonstrated IgE antibodies to α -lactalbumin, β -lactoglobulin, bovine serum albumin, and bovine gamma globulin in most milk-allergic patients (40). However, recent studies utilizing sera from children with milk challenge-confirmed hypersensitivity have suggested that caseins are the most allergenic milk proteins (41–43). In a study of 80 milk-allergic patients (~70% less than 5 years of age), Docena et al. found casein-specific IgE in 80/80 patient sera, β -lactoglobulin-specific IgE in 10/80, and α -lactalbumin in 5/80 (43). Our recent study of 69 milk-allergic children confirmed the importance of casein as a major milk allergen and also demonstrated significant reactivity to α -lactalbumin and β -lactoglobulin. Immunoblotting techniques also have shown cross-reactivity between milk proteins in cows, goats, and sheep. Of 20 children with challenge-confirmed cow's milk allergy, 18 (90%) reacted to goat's milk during a similar challenge (44). Although skin testing to beef is often positive in cow's milk-allergic individuals, most can ingest beef without any adverse effect. A small number of cow's milk-allergic patients have been identified who can eat well-cooked beef, but who experience symptoms with rare beef (45).

Much less information is available about T-cell responses to specific milk proteins. In one study of 34 children with non-IgE-mediated cow's milk allergy, *in vitro* lymphocyte proliferation to casein, α -lactalbumin, and β -lactoglobulin was evaluated (46). Overall, 81% of patients showed a significant response to casein, 21% to α -lactalbumin, and 59% to β -lactoglobulin. The specificity of these responses in defining pathogenic processes is poor, since most normal individuals also demonstrate lymphocyte proliferation following milk-antigen stimulation *in vitro* (47).

Chicken's egg is one of the most common causes of food-allergic reactions in children. The yolk is considered less allergenic than the white (48), although IgE antibodies to chicken gamma globulin, apovitellenin I, and contaminating egg-white proteins can be demonstrated. Egg white contains 23 different glycoproteins, most of which have been purified and their amino-acid sequences determined (49). Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal

d 4) have been identified as major egg allergens (50, 52). Ovalbumin (Gal d 2) is a monomeric phosphoglycoprotein consisting of 385 amino acids and having a molecular weight of 43–45 kDa. Ovotransferrin (conalbumin) comprises 686 amino acids and has a molecular weight of 77 kDa. The full amino-acid sequences of these egg proteins are known and the cDNA clones isolated. Ovomucoid comprises 10% and ovalbumin greater than 50% of the total egg-white protein, and both are easily demonstrable in raw and cooked eggs. Although ovomucoid comprises only a minor component of egg white, we demonstrated that it is the immunodominant allergen (53). More recently, Urisu et al. further demonstrated that ovomucoid is the major allergenic protein in egg white by showing that most egg-allergic children fail to react to ovomucoid-depleted egg white in oral food challenges (54).

Utilizing pooled sera from egg-allergic patients, we identified five IgE- and seven IgG-antibody-binding sites along the 186 amino-acid residues comprising ovomucoid (55). However, evaluation of "linearized" (reduced and alkylated) ovomucoid suggested that not all patients had IgE antibodies recognizing linear ovomucoid epitopes. We then utilized overlapping, linear decapeptides and linearized whole ovomucoid to compare individual patient IgE antibody binding to linear ovomucoid epitopes. When IgE binding to the synthesized decapeptides was compared, it appeared that there were three different patterns of antibody binding. One patient group's IgE antibodies recognized most of the ovomucoid allergenic epitopes previously identified (55), the second group's IgE recognized allergenic epitopes primarily in the first ovomucoid domain, and the third group had virtually no IgE binding to any of the synthesized decapeptides. Since the quantities of egg-specific IgE antibodies were similar in the three groups, this suggested that the third group of patients possessed ovomucoid-specific IgE antibodies that recognized primarily conformational epitopes (56). This supposition was supported by findings comparing the binding of patients' ovomucoid-specific IgE to "native" and "linearized" ovomucoid. While ovomucoid-specific IgE antibody binding to native ovomucoid was similar in the three patient groups, at least 80% of the third group's ovomucoid-specific IgE bound conformational ovomucoid epitopes compared to the first group's ovomucoid-specific IgE antibodies, which demonstrated greater than 50% binding to linearized ovomucoid epitopes.

IgG antibodies to food proteins can be detected in virtually all individuals exposed to food antigens (57, 58), although levels of IgG food-specific antibodies tend to decrease with age (59). Interestingly, when ovomucoid-specific IgG antibody binding to

linear decapeptides was evaluated, significant differences were seen between egg-allergic patient groups and controls. The first patient group, which had the greatest amount of ovomucoid-specific IgE to the linear decapeptides, had significantly less ovomucoid-specific IgG antibody binding to the ovomucoid decapeptides than the second and third patient groups. However, there was no significant difference in the percentage of ovomucoid-specific IgG binding to "linearized" ovomucoid compared to the native form. Control groups consisting of atopic dermatitis patients who were not egg-allergic and nonallergic normal controls had significant IgG antibodies to native ovomucoid but virtually no ovomucoid-specific IgG antibodies to the linear decapeptides. These results suggest a qualitative difference at the level of antigen processing between food-allergic and nonallergic individuals in their immune response to ingested allergens.

Although all egg-allergic patients in this study developed their egg hypersensitivity in the first 2 years of life, the first patient group was older and had more pronounced allergic reactions following the ingestion of egg than the third patient group. The first patient group's extensive ovomucoid-specific IgE antibody binding to numerous linear allergenic epitopes is similar to that seen in patients peanut-allergic to Ara h 1 and Ara h 2 (60). Patients with peanut allergy tend to have "protracted" (lifelong) reactivity to peanut (3), suggesting that "protracted" food hypersensitivity is associated with the development of significant quantities of IgE antibodies to linear epitopes.

In the past decade, much has been learned about the allergenicity of food proteins. Many of the major allergenic proteins have been identified, their amino-acid structure defined, and their cDNA isolated, sequenced, and cloned. While no "allergenic motif" has yet been recognized, further studies defining allergenic fractions and their tertiary structures should lead to a better understanding of allergens in general and should allow us to design recombinant proteins for use in immunotherapy. To date, little work has been done on T-cell epitopes that contribute to allergic responses to foods. Further studies will need to focus on this area if we hope to gain more understanding of how allergic responses develop and are regulated.

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