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Principles and Characteristics of Food Allergens

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I. INTRODUCTION

A. Difficulties Investigating Food Allergens

Much remains to be learned about food allergy. The frequencies, severity, and variety of symptoms caused by foods are controversial, and a precise definition of food allergy is still lacking. Many food-induced sensitivities persist for years, whereas others do not. The target organ specificity of many food allergies is poorly understood and variable from patient to patient. Some foods are more common in provoking allergic reactions than others that are very similar. Still unresolved is whether other mechanisms, in addition to immunoglobulin E (IgE) antibodies, are important in food allergy. The diagnosis of food allergy is still difficult because many food allergen extracts used for skin testing and IgE antibody determinations are neither well characterized nor well standardized. Even the gold standard for the diagnosis of adverse reactions to foods, the double-blind placebo-controlled food challenge (DBPCFC), is only as accurate as the activity of the food material used and the circumstances of the challenge test.^{1,2}

What are food allergens? There have been several immunologic mechanisms implicated as pathophysiologic mechanisms in food allergy. However, IgE-mediated reactions are believed to be responsible for most, if not all, food-induced allergic reactions of the immediate hypersensitivity type.² Food allergens are defined as those food component(s) that induce the production of, and react with, IgE antibodies to cause mediator release from mast cells and basophils resulting in

immediate hypersensitive reactions. This is a very broad definition that includes not only ingested food allergens, but also food aeroallergens and contact food allergens.

Through application of both classical immunochemical methods and recently developed recombinant DNA technologies, considerable knowledge has been obtained about allergen structure and identification of allergenic determinants.^{3,4} Our knowledge of the structure of allergens will continue to increase rapidly primarily due to the cloning and sequencing of allergens. These methods will also provide large amounts of allergenic materials for clinical use.³⁻⁵

B. Special Challenges That Food Allergens Present

Although most food allergens are similar to other allergens that are present in our environment, there are a number of unique features that may make them somewhat different from other allergens. First is exposure. Clearly the majority of exposures to food allergens occur through the gut whose primary function is to process ingested food into a form that can be absorbed for energy and cell growth. This organ presents a rather unusual exposure environment containing a variety of hydrolytic enzymes, acids, and bile salts that can substantially affect the structure of most molecules.⁶ However, some food proteins remain immunologically active, pass through the epithelium, enter the circulatory system, and are distributed throughout the body.⁷⁻¹⁴ The factors that affect food digestion and result in absorption of intact allergens require better definition. Further-

more, the digested allergen may be different from the form that was originally ingested. This further complicates establishment of a cause-and-effect relationship necessary for allergen identification.

The gut-associated lymphoid tissue (GALT) is unique in that it must mount a rapid response to harmful foreign substances while remaining unresponsive to enormous quantities of nutrient antigens. GALT is composed of Peyer's patches and the appendix, lymphocytes and plasma cells of the lamina propria, intraepithelial lymphocytes, and mesenteric lymph nodes.¹⁵ It may respond differently from peripheral lymphoid tissue in the spleen and lymph nodes from which most of our information concerning the immune response is obtained. Food antigens in such an environment may have different properties, compared with conventional antigens.

Exposure to foods also occurs through other routes such as through contact (skin) and inhalation (lungs). This has been well described in occupational reactions in food industry workers.¹⁶ The relationship of food allergic responses induced through inhalation to those stimulated through the gut and the similarities and/or differences of allergens that induce such responses need to be studied. There is also a need to establish the relationship of inhaled allergens to ingested allergens with regard to food allergy pathogenesis, and to determine other significant factors about food allergies such as threshold levels of sensitization for provocation, important in terms of establishing parameters for regulation.¹⁷

C. Complexity of Foods

Food is a complex mixture containing a variety of allergenic and nonallergenic components. Identifying, purifying, and characterizing the allergens in food can be a major challenge. Most known food allergens are soluble in aqueous solvents; there is essentially no information on allergenic components that are not soluble in aqueous solvents. As noted above, digestive processes can also affect food allergens. The nature of food allergens that actually interact with the GALT may be quite different from the form that was ingested with foods.

Some food allergens may be present in trace amounts, yet may be major allergens. Purifying and characterizing such trace allergens can be especially difficult. Because of the exquisite sensitivity of some allergic consumers, allergens may be present in harmful amounts even as trace contaminants. This situation can easily lead to mistaken conclusions regarding the allergenicity of specific foods.

Significant allergenic cross-reactivity has been described among foods from closely related sources as well as between foods and seemingly unrelated or remotely related materials with essentially no phylogenetic relationship.^{18,19} Legumes^{19,20} and crustacea¹⁹ are examples of foods that cross-react within the same family. However, the mere fact that members of these food families cross-react does not necessarily mean that these reactions are clinically significant. The substantial cross-allergenicity for crustacea appears to be clinically important,^{21,22} but cross-reactivity among the legumes does not appear to be clinically significant.²³ These observations further complicate our understanding of food allergen reactivity.

In an attempt to organize allergens with regard to patient reactivity, the following classification system has been developed to better describe allergens. Allergens to which the majority of patients react are described as "major allergens," compared to other allergens, or "minor allergens," to which a minority of patients react²⁴ (see Article 7). Generally, although not always, major allergens tend to be predominant components present in allergen extracts. There have been several attempts to define major allergens based on the number of individuals who react with these materials, or on the intensity of the biological reaction.²⁵⁻²⁷ Although major allergens are generally of greatest interest, we cannot ignore minor allergens, which also sometimes cause serious reactions in sensitized subjects (see Article 7).

Some extracts contain allergens, which although having similar physical, chemical, and immunochemical structures, differ slightly from one another in isoelectric point.²⁴ The term *isoallergens* has been used to denote such molecules isolated from the same source. The slight differences observed in these allergens are due to minor structural differences in the carbohydrate

moiety of the molecule (degree of glycosylation), differences in degree of protein amidation, or genetic variation.²⁸

II. PROPERTIES OF FOOD ALLERGENS

A. General Characteristics of Allergenic Food Proteins

Foods contain a variety of different proteins, only a few of which are known allergens. In plant foods such as soybeans and peanuts, many of the allergenic proteins are storage proteins²⁹⁻³¹ that exist in large amounts in some food. It might be tempting to conclude that the likelihood of allergenicity is correlated with the extent of exposure to a particular protein. However, proteins present in less significant or even minor amounts also serve as major food allergens. For example, *Gad c 1*, the major allergen in codfish, is not a particularly prominent protein.³² Also, many major proteins such as actin and myosin in beef, pork, and chicken are not allergenic. Although the dose of exposure is likely a critical factor, the immunogenicity of the protein is clearly more important.

As with all antigens, to stimulate a significant immune response, food allergens must be foreign molecules to the host, because molecules of host origin are not normally recognized by the immune system. Most food allergens are highly stable molecules that are resistant to food processing and cooking, and the digestive processes.³³ There are, however, exceptions such as the labile allergens present in apple and other fresh fruits and vegetables.^{34,35} Generally, such labile allergens cause reactions that are restricted to the oral mucosa, as they lose allergenic activity after degradation. Conversely, digestion may alter antigens by releasing or uncovering neoepitopes that induce allergic reactions, although this possibility has not been well investigated.

Historically, mucosal permeability has been thought to be a major factor in food allergy. Many food proteins cross the gut mucosal membranes, prompting immune responses even in normal individuals.^{13,36} However, differences in mucosal absorption of allergens have not been well stud-

ied in food-allergic individuals, compared with normal individuals; the relationship of the phenomenon of gut permeability to food allergy has not been resolved either. This is a particularly important issue in infants prior to mucosal gut barrier maturation when large molecules more easily pass through the gut epithelium and induce allergic sensitization.⁶

Finally, there is the nature of the food protein being ingested. Some foods that are common components in our diets are not very allergenic. For example, beef and pork are important foods in the American diet that have a high protein content. Yet, beef and pork are not common causes of food allergy, compared with fish and shellfish, which are major allergenic foods. This is particularly intriguing because beef and pork also contain the muscle protein tropomyosin, a molecule identified as the major allergen in shrimp.³⁷⁻⁴⁰ Although chicken and shrimp tropomyosin share significant (60%) amino acid sequence homology, the allergenic reactivity differs considerably. Shrimp tropomyosin and presumably tropomyosins present in other crustacea are very potent allergens, whereas tropomyosins present in beef, pork, and chicken are not. Structural differences that contribute to or diminish the allergenicity of a molecule are important topics for further research.

B. Biochemical and Immunochemical Characteristics of Allergenic Food Proteins

Because all allergens must be able to bridge IgE molecules on the surface of mast cells to cause degranulation, they are somewhat constrained in their molecular dimensions. Thus, these allergens must contain at least two IgE antibody-reactive sites (B-cell epitopes) to trigger mediator release. However, it is noted that some monovalent allergens, such as the 21-residue venom peptide mellitin,⁴¹ still elicit histamine release from basophils or mast cells or generate anaphylaxis in mice. These molecules have the ability to bind to IgE antibodies on the surface of basophils/mast cells. They aggregate, or aggregate and then bind, converting the monovalent allergens to polyligands

that can cause allergic reactions. They also act as haptens by binding to macromolecules and subsequently as complexes that cause IgE bridging. It has not yet been determined that this occurs *in vivo* in allergic disease and is a substantial factor in allergic reactions.

Most known food allergens have mol wt between 10,000 and 70,000 Da.¹⁹ Although smaller molecules may be immunogenic, the mol wt of 10,000 Da probably represents the lower limit for the allergenic response. The upper limit is probably a result of restricted mucosal absorption of larger molecules.¹⁹ However, some allergens such as the peanut allergens *Ara h 1* (mol wt 63.5 kDa) and *Ara h 2* (mol wt 17 kDa),^{42,43} exist in native form as large protein polymers that are 200 to 300 kDa in size. It is not clear if such large molecules act as allergens or are disassociated during the digestive process. Most food allergens are glycoproteins with acidic isoelectric points. However, this is a property of most antigens and does not necessarily represent a unique property for food allergens. All allergens are proteins, but not all proteins are allergens. Many allergens contain posttranslational modifications, such as the addition of oligosaccharides.

The biochemical structure of a few food allergens has been determined (see "Food Allergens" by Robert K. Bush and Susan L. Hefle in this issue). When the biochemical structure of various allergenic proteins is compared, there does not appear to be any consistent pattern that is representative of allergens in general, or food allergens specifically. Comparisons of primary amino acid sequences of allergenic proteins have not revealed unique or typical patterns. There also does not appear to be any particular pattern to the protein tertiary structure, such as an alpha helix, beta strand, or loops.⁴⁴ When the primary structure of an allergen is compared to other proteins, amino acid sequence similarities occur with a number of proteins in our environment. In light of the evolution of all living organisms, this should not be surprising, but illustrates that there may be certain as yet undetected structural features of allergens that render them different from other proteins.

The surface of an antigen/allergen that interacts with cells of the immune system or specific antibodies are defined as epitopes. Epitopes that

react with T cells are called T-cell epitopes.⁴⁵ Those that react with antibody or antibody-producing B cells are called B-cell epitopes.⁴⁴

Epitopes are either conformational or linear. Conformational epitopes depend on the tertiary structure of the protein or several amino acid sequences on the protein surface. Linear epitopes depend on the linear sequence of amino acids in a protein. The general belief is that T-cell epitopes are linear, whereas B-cell epitopes are conformational. However, there are also exceptions to this rule.⁴⁴ By definition, all B-cell epitopes must be on an outer surface of the molecule (Figure 1). If an epitope is composed of a series of covalently linked amino acids it is called a continuous epitope. An epitope that is composed of two different amino acid sequences which through their tertiary structure form one epitope is called a discontinuous epitope. Because investigating discontinuous epitopes is technically more demanding, there is generally much more information available on continuous epitopes. The minimum number of amino acid residues is 8 for continuous epitopes,⁴⁶ whereas discontinuous conformational epitopes have 16 or more.⁴⁴

T cells exclusively recognize protein antigens that are processed by proteolysis into short peptides. They are recognized by the T-cell receptor (TCR) only after the allergen has been phagocytized by an antigen-presenting cell (APC).⁴⁶ By structural analysis with X-ray crystallography, it has been shown that the peptides are set in an extended form in a cleft of the presenting molecules between the α -helices of the α_1 and the α_2 domain.⁴⁶ Antigen processing by an APC includes proteolytic digestion of the antigen and presentation of the peptide fragments in the peptide binding groove of the HLA class II molecule on the APC surface. The TCR then responds specifically to the epitope in the major histocompatibility complex (MHC) II groove. Chemical analysis of the peptides eluted from MHC molecules has established that they are usually eight to nine amino acids long.⁴⁶ Determination of the complete repertoire of T-cell epitopes of allergens remains a formidable task due to their number and the variability in recognition by different individuals.

Although our knowledge of T-cell epitopes of food allergens is largely extrapolated from

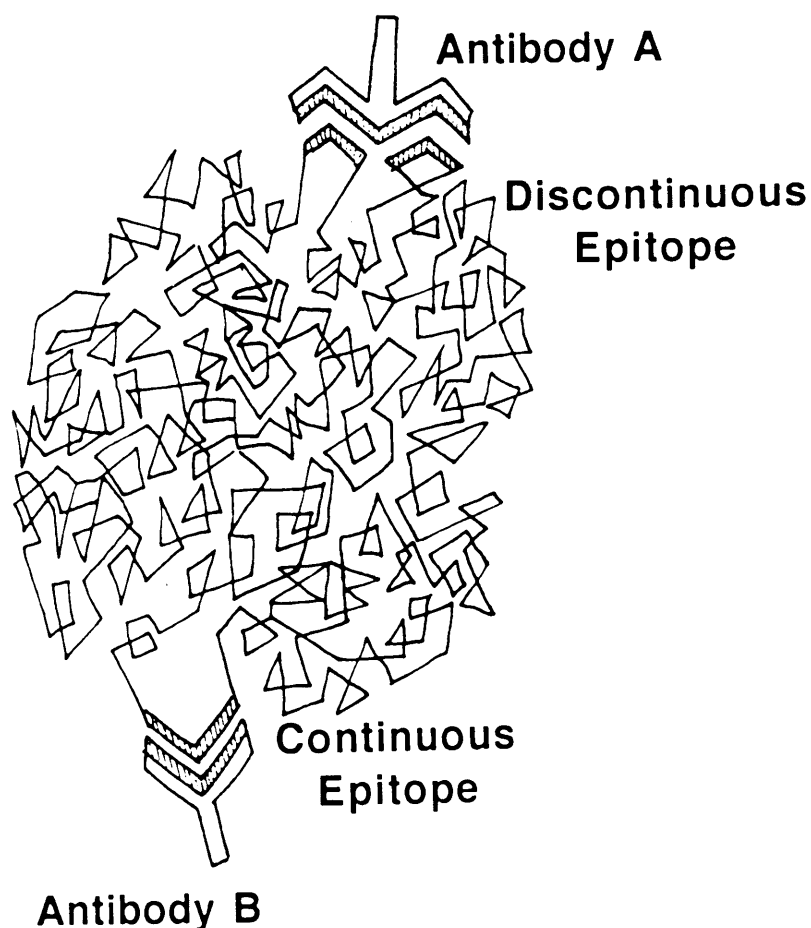


FIGURE 1. The epitope structures of allergens.

nonfood sources, such as pollens, food allergens may have unique properties. Food allergens are recognized by GALT or other lymphatic cells in the body. The immunoregulatory mechanisms of T-cell responses of these systems have not been well defined; thus, there may be unique properties to T-cell epitopes in food allergens. Identification of T-cell epitopes may be achieved using overlapping peptides based on their ability to induce responses in T cells present in peripheral blood mononuclear cells or in T-cell clones derived from individuals sensitized to the native allergen. However, because a substantial amount of blood must be readily available from donors, these data are restricted by the availability of source material. On the basis of inducing nonresponsiveness and in the down-regulation of established immune responses in animals, there is considerable interest in peptides containing T-cell epitopes as a

basis for immunotherapy.⁴⁷ Immunotherapy with T-cell-reactive peptides is under intense study.

B-cell epitope peptides do not appear to have any unique or common patterns in amino acid sequences (Table 1). Antibodies specific for continuous epitopes invariably have a lower affinity than those for discontinuous epitopes.⁴⁴ From the point of view of antibody affinity, most antibodies produced by an allergic person appear to be specific for discontinuous (high affinity) epitopes.⁴⁴ Whether this generalization applies to food allergens is not yet known (Table 2). Although there are approximately 8 to 16 residues in an epitope, not every single residue contributes equally to the total binding energy.^{44,46}

The entire surface of a protein antigen is covered with potential B-cell epitopes; however, they are all not as equally reactive. In fact, many are not recognized. There are methods to predict B-cell

TABLE 1
Sequence of Some B-Cell Epitopes of Five Allergens

Source	Allergen/residue	AA sequence	Ref.
Ragweed pollen	<i>Amb a 3</i> residue 53–63	TrpArgGluGluValArgAsnGluGluAlaTyr	47a
Mite	<i>Der p 1</i> residue 117–133	CysGlnIleProProAsnAlaAsnLysIleArg GluAlaLeuAlaGln	47b
Bee venom	<i>Api m 4</i> residue 20–26	IleLysArgLysArgGlnGln	47c
Milk	Lactoglobulin residue 124–134	ArgThrProGluValAspAspGluAlaLeuGlu Ala	47d
Shrimp	Tropomyosin residue 153–161	PheLeuAlaGluGluAlaAspArgLys	39

TABLE 2
Continuous and Discontinuous B-Cell Epitopes of Some Food Allergens

Source	Name	Antibodies tested	Continuous epitopes	Discontinuous epitopes	Ref.
Egg	<i>Gal d 2</i>	IgE + IgG	7		47e
	<i>Gal d 4</i>	IgG		3	47f
Milk	β -lactoglobulin	IgE	1		47d
	α -lactalbumin	IgE	1		
Wheat	Amylase inhibitor	IgE	5		47g
Fish	<i>Gad c 1</i>	IgE + IgG	3		47e
Shrimp	<i>Pen a 1</i>	IgE	2		39

epitopes, although they are not totally successful. For example, algorithms based on the calculations of polar and nonpolar amino acid residues have been used to predict those residues located on the surface of the molecule. However, this information is incomplete because host responsiveness determines those epitopes to which an individual reacts.

C. Agronomic Considerations

Variations may occur in the type and amount of individual proteins in foods as a result of agronomic conditions. For example, the glycinin content of soybeans⁴⁸ and the arachin content of peanuts⁴⁹ are affected by agronomic conditions. However, no studies have been done to determine if such conditions have any effect on the allergenicity of specific foods.

The type and amount of individual proteins in foods may also vary from one variety or cultivar to another. Very limited studies have been conducted to determine if such conditions have any effect on the allergenicity of specific foods. The IgE-binding proteins of almonds are similar for the three major varieties: Nonpareil, Mission, and Carmel.⁵⁰ Florunner and Virginia peanuts are reasonably equivalent in their abilities to bind peanut-specific IgE in inhibition immunoassays.⁵¹ Some individuals with allergies to fresh avocados react to only one of two common varieties, although most react to both varieties.⁵² The amount of a specific 27-kDa albumin in wheat, which is a major occupational allergen in baker's asthma, was variable among seven wheat cultivars used commonly in Germany.⁵³

The stage of development of a plant or animal also affects the nature and amount of specific proteins and perhaps allergens. Again, this possi-

bility has received little study. Soybean sprouts are equivalent to soybean seeds in their ability to bind IgE from soy-allergic patients in inhibition immunoassays.⁵⁴

D. Physical and Chemical Characteristics of Allergenic Proteins

Food allergens tend to be resistant to typical processing and preparation conditions. These proteins are comparatively resistant to heat and acid treatment, proteolysis, and digestion. However, important exceptions do exist. Although these resistance properties may be useful in the assessment of the potential allergenicity of a specific protein of unknown allergenic potential that has been transferred or developed through genetic engineering, a combination of tests should be used to increase the reliability of such evaluations.

1. Resistance to Heat

Many allergenic food proteins are resistant to heat. However, some heat-labile food allergens have been identified. The application of heat promotes protein denaturation and the loss of conformational IgE-binding epitopes. The resistance of some food allergens to heat denaturation suggests that conformational epitopes are not always critical for IgE binding in the case of food allergens. Cow's milk proteins have been the most extensively studied in this regard. Heat treatment can reduce the antigenicity of whey proteins, but has virtually no effect on the antigenicity of casein.⁵⁵ However, whey proteins retain some allergenicity even when subjected to severe heat.⁵⁵⁻⁵⁷ The IgE-binding capacities of β -lactoglobulin and bovine serum albumin (BSA) are diminished by heating at 80 to 100°C for 15 min. In contrast, little change is observed in the IgE-binding capacities of α -lactalbumin and the caseins after heat treatment.⁵⁷ The IgE antibodies in the sera of some patients still react significantly with milk proteins, even after severe heating at 100°C for 3 h.⁵⁷ This heterogeneous response of patients with cow's milk allergy to heat-treated milk proteins illustrates the

difficulties of using heat treatment as a hypo-allergenic process.⁵⁷

Most other food allergens are also resistant to heat. The primary codfish allergen, *Gad c 1*, is resistant to heat.⁵⁸ The major allergens in shrimp are heat stable⁵⁹⁻⁶¹ and have been isolated from the cooking water.^{60,61} The IgE-binding ability of the rice glutelin and globulin fractions as assessed by immunoassay was reduced by 40 to 70% by heating at 60°C for 1 h or 100°C for 2 to 10 min.⁶² The 7S- and 11S-globulin fractions of soybean bind soy-specific IgE, but that binding activity can be decreased but not eliminated by heating at 80°C for 30 min.⁶³ Later, Burks et al.³⁰ demonstrated that the IgE titers from the sera of patients with soy allergy were reduced when examined using the 7S and 11S fractions of soy protein that had been heated at 80°C for 60 min by comparison to unheated soybeans. No change was observed in the IgE titer using the whey fraction of soy protein under those conditions. Substantial decreases in the IgE titers were observed when examined with the three soy protein fractions after heating at 120°C for 60 min.³⁰ In further experiments, the IgE-binding capabilities of the three major soy protein fractions were only minimally affected by heating at 37°C for 60 min, 56°C for 60 min, 100°C for 5 min, 100°C for 20 min, or 100°C for 60 min.³¹

The IgE-binding capabilities of a crude peanut extract and two major peanut allergens, *Ara h 1* and *Ara h 2*, were unaffected by heating under similar conditions.³¹ The IgE-binding ability of one of the major peanut allergens, the concanavalin A-reactive glycoprotein, is stable to temperatures of up to 100°C over a pH range of 2.8 to 10.⁶⁴ Raw peanuts also appear to contain several heat-labile allergens.⁶⁵ The allergenic activity of the albumin fraction of peas retained all of its activity when heated or boiled.⁶⁶ The allergens in cottonseed are quite heat stable.¹⁸ Ovalbumin and ovomucoid, two of the major allergens from egg whites, are relatively heat stable and can be immunologically detected in cooked eggs, while conalbumin is less stable.⁶⁷ Ovomucoid retains its ability to bind to IgE from the serum of egg-allergic patients even after prolonged heating at 100°C, although the ovomucoid from eggs stored for 12 d at 4°C was slightly less stable to heating

at 100°C than ovomucoid from fresh eggs.⁶⁸ Thermal denaturation of ovalbumin had little effect on its allergenicity⁶⁹ in one study, but heat-denatured ovalbumin had substantially less binding to IgE when compared with native ovalbumin in another study.⁷⁰

Some food allergens are quite sensitive to heat denaturation. The allergens in fresh fruits and many vegetables are a good example.³⁵ With apples, skin prick tests were positive with extracts of fresh apples, but were negative with extracts of apples heated at 175°C for 30 min.³⁴ Other foods also contain a mixture of heat-stable and heat-labile allergens; peanuts as discussed are a good example.⁶⁵

Although food allergens tend to be resistant to heat denaturation, this property is obviously not universal. The use of heat denaturation in the assessment of the potential allergenicity of a specific protein obtained through genetic engineering is problematic. The ability of the heat-treated protein to induce immunogenic responses should be evaluated in IgE-responsive systems. Assessment of the heat stability of other biological activities of specific proteins cannot be used to predict the heat stability of immunogenic or allergenic responses.

2. Resistance to Digestion, Proteolysis, and Hydrolysis

Most food allergens are resistant to proteolysis or hydrolysis.⁷¹ Although heat treatment leads to protein denaturation and loss of conformational epitopes, enzymatic or acidic cleavage of the polypeptide chains may destroy both conformational and linear epitopes. However, the amount of information on the stability of food allergens to digestion, proteolysis, and hydrolysis is relatively limited.

A few studies have even suggested that digestion might enhance the allergenicity of food proteins. Haddad et al.⁷² reported that the IgE from the serum of some patients with cow's milk allergy is actually more reactive with proteolytic digests of β -lactoglobulin than to its undigested form. Schwartz et al.⁷³ reported that pepsin digestion did not diminish the IgE-binding ability of β -lactoglobulin.

Limited proteolytic digestion of cow's milk proteins does not diminish their ability to bind to IgE, although complete hydrolysis does result in a substantial loss of allergenicity.^{74,75} The hydrolysis of whey proteins with trypsin resulted in a partial hydrolysate that had no sensitizing capacity in guinea pigs.^{76,77} A commercial infant formula made with this hydrolysate was determined to contain less than 20% free amino acids, detectable peptides with chain lengths of up to 10 to 15 amino acids, and a small amount (about 1%) of proteins with mol wt up to 3000 indicating the presence of proteins of 27 amino acids in length.⁷⁸ These proteins are apparently reactive with cow's milk specific IgE based on the occurrence of adverse reactions to this formula among milk-sensitized infants^{79,80} and the demonstration of significant IgE binding to these partial whey hydrolysates using the sera of certain cow's milk-allergic subjects.⁸¹

The combined effects of pepsin and trypsin on whey proteins resulted in a greater degree of hydrolysis and abolished the binding to IgE.⁸² The hydrolysis of whey proteins has also been conducted with an alkaline protease from *Bacillus licheniformis* and the resulting peptides separated according to their mol wt by gel filtration.⁸³ The peptides having mol wt above 6 kDa were capable of eliciting local hypersensitivity reactions in mice while the smaller peptides did not,⁸³ suggesting that extensive hydrolysis may be necessary to eliminate allergenic peptides derived from whey proteins. The partial hydrolysis of whey proteins with a mixture of commercial proteases produced two hydrolysates with 20.8 and 12.4% degrees of hydrolysis, respectively.⁸⁴ Although these hydrolysates displayed substantially reduced IgE binding, their IgE reactivity was not completely destroyed.⁸⁴ Ultrafiltration of the hydrolysates did produce a hydrolyzed whey permeate with no detectable IgE-binding activity.⁸⁴

The effect of digestion and partial hydrolysis of casein on its allergenicity in humans has not been investigated. In rabbits, casein peptides with mol wt between 3500 and 5000 were as immunogenic as the intact protein, whereas smaller peptides were 1000-fold less immunogenic.⁸⁵ While partial casein hydrolysates tend to retain some immunogenicity, the specificity of the proteolytic enzymes used in the generation of these hydroly-

sates has some effect on residual immunogenicity.⁸⁶ The extensive hydrolysis of casein does yield a product with greatly diminished allergenicity that is commonly used to produce a hypoallergenic infant formula.^{75,87} Careful examination of these casein hydrolysates revealed that they contained 70% free amino acids and detectable peptides with chain lengths up to five to eight amino acids.⁷⁸ IgE binding to casein hydrolysates is also detectable in some patients.⁸¹

Other food allergens are also resistant to proteolysis and digestion. The codfish allergen, *Gad c 1*, is resistant to proteolysis; small peptide fragments of *Gad c 1* retain their ability to bind to IgE.⁸⁸⁻⁹⁰ However, extensive hydrolysis of a codfish allergen extract with trypsin, pepsin, subtilisin, and pronase resulted in the destruction of its IgE-binding ability. Elastase hydrolysis and simulated digestive proteolysis were only partially effective.⁹¹ Similarly, the IgE-binding abilities of ovomucoid and ovalbumin, the major allergens in egg white, were also unaffected by proteolysis and resulted in the isolation of a peptide fragment with IgE-binding activity.^{69,70,92}

The IgE-binding capability of a soybean extract was reduced tenfold when subject to pepsin hydrolysis followed by hydrolysis with trypsin, chymotrypsin, and a mixture of intestinal peptidases.³¹ When subjected to similar proteolytic treatment, the IgE-binding capability of a peanut extract was reduced 100-fold.³¹ The extensive hydrolysis of soy protein yielded a hydrolysate that was not immunogenic to rabbits.⁹³ Similar results were obtained with guinea pigs using a collagen/soy protein hydrolysate.⁹⁴

The skin test reactivity of wheat extracts in a group of patients with IgE-mediated, exercise-induced anaphylaxis was destroyed by treatment with trypsin, but not with pepsin.⁹⁵ The sizes of the peptides generated by trypsin and pepsin hydrolysis were similar. However, partial hydrolysis with trypsin destroyed the IgE-binding epitopes on wheat proteins, whereas pepsin hydrolysis did not materially affect these same epitopes.⁹⁵ A hypoallergenic wheat flour has been developed by treating solubilized wheat proteins with actinase, collagenase, and transglutaminase.⁹⁶

The major cottonseed allergens are unaffected by pepsin hydrolysis.⁹⁷ The rice allergen could be largely inactivated by hydrolysis with actinase,

while papain caused a decrease in the IgE-binding activity of the rice allergen. Pepsin, trypsin, chymotrypsin, and pancreatin had no effect on the activity of this allergen.⁹⁸

Proteins may also be hydrolyzed by chemical means, either through specific agents such as cyanogen bromide or nonspecific methods such as treatment with concentrated hydrochloric acid. Chemical modifications of allergens may offer another means for the reduction of the allergenicity of these proteins. Cyanogen bromide cleavage of ovomucoid had no effect on its IgE-binding ability.^{92,99} However, this treatment reduced the allergenicity of ovalbumin.^{69,99} The denaturation of ovalbumin with urea, hydrochloric acid (pH 3.0), and dithiothreitol did not diminish its IgE-binding activity, but treatment with sodium hydroxide (pH 11.0) did destroy its IgE-binding activity.⁷⁰

Some food allergens are more sensitive to proteolysis and digestion. Fresh fruits commonly cause oral allergy syndrome (OAS) in affected individuals.^{100,101} Apparently, such allergens in fresh fruit are easily digested in the gastrointestinal (GI) tract and do not cause systemic effects. This theory, however, has not been carefully evaluated.

Most food allergens are resistant to digestion, proteolysis, and other forms of hydrolysis. However, the findings depend on the nature of the enzymatic or chemical treatment used in the experiments, the choice of method for assessing the immunogenicity of the hydrolysis products, and, to a lesser extent, the specific food allergen being evaluated. Although the assessment of the resistance to hydrolysis of proteins could offer valuable information regarding the potential allergenicity of specific proteins, a rigorous protocol for such experiments has not been established. Ideally, this protocol would mimic digestive proteolysis and include tests on the isolated protein and the protein in the appropriate food matrix. The experience with the evaluation of the immunogenicity of partial whey hydrolysates in animal models dictates that extreme caution be used in the evaluation of results obtained from such animal models. The development of further data on the comparative stability of food allergens vs. other food proteins to digestion, proteolysis, and hydrolysis would be highly desirable to deter-

mine the utility of this tool for the assessment of the potential allergenicity of specific proteins.

3. Solubility

Most food allergens are soluble in water and/or saline solutions, thus belonging to the classes known as albumins (water soluble) and globulins (salt soluble).¹⁰² However, many food proteins fall into these two solubility categories, so these features are not particularly distinguishing. Food proteins may also be either alcohol soluble (prolamines such as gliadin from wheat) or alcohol insoluble (such as glutenin from wheat). Proteins in these two categories have not been frequently classified as allergens. However, these two categories of proteins have been less frequently examined for their allergenic potential. Occasionally, these proteins have been implicated in IgE-mediated allergies. For example, IgE antibodies to wheat gluten have been identified in a few wheat-allergic patients.¹⁰³ Also, celiac disease, which involves a cell-mediated immune reaction associated with an intolerance to wheat, rye, barley, and oats, is caused by certain gliadin peptides.¹⁰⁴ Recently, a codfish allergen was identified in codfish surimi from which the soluble proteins, including *Gad c 1*, had been removed by extensive washing.¹⁰⁵

4. Acid Stability

Food allergens are usually quite stable to moderate acid treatments.³³ The treatment of food allergens with acid concentrations simulating stomach acid conditions typically has little effect. The lability of the allergens in fresh fruit to such acidic conditions has already been noted as an exception to this statement. More typically, one of the major peanut allergens, the 65-kDa conavalin A-reactive glycoprotein, is stable at pH 2.8.⁶⁴ Ovalbumin, one of the major eggwhite allergens, is stable at pH 3.0.⁷⁰ A demonstration of a lack of immunogenic stability under simulated stomach acid conditions could be an additional criterion to apply in the assessment of the potential allergenicity of a given protein. Again, rather

limited information exists on the comparative acid stability of food allergens. Gathering additional data would be an important consideration.

5. Effect of Chemical Modification of Amino Acids on Allergenicity

Chemical modification may also involve the modification of the amino acids in the IgE-binding epitopes, decreasing binding to allergen-specific IgE. These approaches occasionally have value in identifying the amino acids involved in the IgE-binding epitopes, but do not generally have much practical value in the processing of foods. In one of the earliest attempts to use this strategy, Lee and Sehon¹⁰⁶ observed a decrease in the IgE binding of ovalbumin after treatment with polyethylene glycol. The chemical modification of the lysyl, tyrosyl, tryptophyl, or arginyl side chains of *Gad c 1*, or the release of the two calcium ions associated with this protein also resulted in a significant reduction in IgE binding.¹⁰⁷ The polymerization of *Gad c 1* similarly resulted in diminished allergenic activity.^{107,108}

Other approaches also diminish the IgE binding of some food allergens, but these are the exception rather than the rule. In contrast, the reaction of lactose with β -lactoglobulin increased the skin reactivity of this milk protein in cow's milk-allergic individuals,¹⁰⁹ suggesting that the browning reaction may enhance allergenicity in some situations.

The effect of chemical modifications on the allergenicity of various food proteins is likely to be highly specific and related to the amino acid sequence in the epitope of the allergen. The use of such approaches in the general assessment of the potential allergenicity of genetically engineered proteins would be impossible.

III. EFFECT OF PROCESSING ON FOOD ALLERGENS

A. Thermal Processing

Because food allergens are generally resistant to heat and proteolytic enzymes, food processing

is not particularly effective in eliminating the allergenicity of various foods. Some notable exceptions do exist. For example, the allergenicity of fresh fruits is rather easily eliminated by heating,¹⁰¹ such as would occur in the canning of fruits or the making of jam. The allergenicity of sliced apples is diminished by storage at room temperature,^{34,110-112} probably another indication of the ease of denaturation of some of the allergens found in fresh fruits. However, the allergens in freshly picked apples increase during storage if the apples are not sliced.¹¹² The allergens present in salmon seem to be destroyed by the thermal processes used in the commercial canning of salmon because salmon-allergic patients in one study could tolerate canned salmon.¹¹³ However, fish-allergic individuals are often allergic to cooked fish, so the extent of the heat process is an important variable.

Some occupational food allergens are heat labile. Green coffee beans were found to possess allergenic activity, but roasted coffee beans did not, which suggests the presence of heat-labile allergens.¹¹⁴ The cocoa bean allergen is destroyed by processing into chocolate, but the responsible processing operation has not been identified.¹¹⁵ Because cocoa beans undergo substantial heat processing, the possibility of heat denaturation seems likely, perhaps in concert with chemical modifications.

Most allergenic foods retain their allergenicity during the typical heat processes used in manufacturing or preparation. Cow's milk and its two principal protein fractions, casein and whey, retain their allergenicity even when subjected to severe heat treatments.^{55,116-119} These severe heat treatments may adversely affect the nutritional quality of the milk.¹¹⁶ Cow's milk reportedly retains its allergenicity after such common heat-processing treatments as condensation, evaporation, and drying.¹¹⁹

Both raw and roasted peanuts are allergenic and bind to peanut-specific IgE.^{51,65} Most other commercially processed peanut products, including peanut butter and various peanut flours, retain their allergenicity.⁵¹ Raw and roasted soybeans contain similar IgE-binding proteins.²⁹ Soy protein isolate, which is prepared by acid and alkaline extraction of defatted soybeans, has dimin-

ished IgE-binding capability, although some residual allergenicity remains.²⁹ Other soy products, including soy flours and soy protein concentrate, which were also subjected to drying, retained most of their IgE-binding proteins.²⁹ Boiled eggs retain their ability to bind to IgE.⁶⁸

B. Enzymatic Proteolysis

Enzymatic proteolysis is not typically effective in eliminating the allergenicity of proteins in various allergenic foods. The degree of proteolysis is very likely an important variable, but rather extensive proteolysis may be necessary to eliminate all of the allergenicity of an allergenic food protein. Regulatory agencies typically do not have regulations that differentiate between extensively and partially hydrolyzed proteins. Cow's milk is the best studied example by far. An infant formula was developed based on the partial hydrolysis of whey proteins,¹²⁰ as described by Jost and co-workers.^{76,77} This product was less sensitizing than a typical cow's milk formula when fed to newborn infants in some studies,¹²¹⁻¹²³ but no difference was noted in other studies.^{124,125} This partial whey hydrolysate formula triggered allergic reactions when fed to certain infants already sensitized to cow's milk.^{79,80,126} Complete hydrolysis of an allergenic protein to its constituent amino acids would be predicted to destroy the IgE-binding capability of the protein. In fact, the most widely marketed hypoallergenic infant formulas are based on casein hydrolysates that were subjected to extensive hydrolysis. However, occasional allergic reactions are even reported to casein hydrolysate formula in highly sensitive infants,¹²⁶⁻¹²⁸ which is notable because the degree of hydrolysis in such formulae is greater than 85%.^{93,129} Wahn et al.⁸⁷ determined that the casein hydrolysate formulas indeed had little residual allergenic activity. However, casein hydrolysates can, at least occasionally, contain peptide fragments large enough to be immunogenic.^{83,130}

An extensively hydrolyzed peanut protein sold as a flavor enhancer did not bind to peanut-specific IgE.⁵¹ However, both a commercial- and an acid-hydrolyzed vegetable protein (HVP) made from soybeans bound to serum IgE from soy-

bean-allergic individuals,⁵⁴ probably because the degree of hydrolysis was insufficient to destroy the IgE-binding epitopes on the soybean allergens. Fermented soy products, such as mold-hydrolyzed soy sauce, tempeh, tofu, and miso, also retained their allergenicity, although the level of IgE-binding ability was diminished by comparison to extracts of raw soybeans.⁵⁴

A hypoallergenic wheat flour has been developed through the treatment of solubilized wheat proteins with actinase, collagenase, and transglutaminase.⁹⁶ Similarly, a hypoallergenic rice has been developed through the treatment of rice with proteolytic enzymes.⁹⁸ This preparation was quite effective in the treatment of rice-allergic patients.^{131,132} The selective digestion of β -lactoglobulin from a whey protein concentrate was accomplished by using thermolysin under high hydrostatic pressure (1000 to 3000 kg/cm²). The resultant product was devoid of β -lactoglobulin, but was enriched in α -lactalbumin, another common cow's milk allergen.¹³³

C. Oil Extraction

Removal or exclusion of either all proteins or only the allergenic proteins during processing would obviously eliminate the allergenicity of a resulting food. The extraction of edible oils from oilseeds, such as peanut, soybean, and sunflower seeds, by the hot solvent extraction procedure eliminates all traces of protein. This makes the resultant oils safe for consumption by allergic individuals.¹³⁴⁻¹³⁶ However, Porras et al.¹³⁷ identified soy protein in some, but not all, samples of soybean oil from Europe, indicating that oil extraction may not always eliminate the proteins or the allergens entirely. Soy protein was also detected frequently in samples of soya lecithin, a common food ingredient.¹³⁷ Similarly, nonprotein ingredients from cow's milk may contain traces of the cow's milk allergens. For example, lactose can contain allergenic contaminants.¹³⁸

D. Other Processes

Other approaches may diminish the IgE binding of some food allergens, but these tend to be

the exception rather than the rule. The apple allergen is inactivated by phenolic browning.¹¹⁰ Lyophilization may diminish the allergenicity of certain fish extracts. Fish-allergic patients reacted positively to open challenges with nonlyophilized, cooked fish but negatively to double-blind, placebo-controlled challenges with lyophilized cooked fish.³² However, lyophilization is a fairly unusual food processing operation, and freeze-dried fish are seldom marketed. *Gad c 1* appears to be removed from codfish along with other soluble sarcoplasmic proteins in the production of surimi.¹⁰⁵ (Surimi is minced and extensively washed fish which has a gelatinous nature.) However, another codfish allergen remains in the surimi, making this product nonhypoallergenic.¹⁰⁵

The effect on food allergenicity of other processes used in food manufacturing has not been evaluated. Homogenization has no noticeable effect on the allergenicity of cow's milk.¹¹⁷ This is despite theories that homogenization results in an enhancement of availability of allergenic epitopes on cow's milk proteins. Most allergenic food proteins likely retain their allergenicity during food processing and preparation.

E. Approaches to Hypoallergenic Processing of Foods

Because of the lack of research and developmental interest, little progress has been made in formulating hypoallergenic foods. The only widely commercialized, hypoallergenic foods are certain infant formulas intended for babies who develop allergies to cow's milk and soy proteins used in formulas. These hypoallergenic infant formulas are based on extensively hydrolyzed casein and/or whey. Because the proteinaceous allergens are hydrolyzed to amino acids and very small peptides, these formulas are relatively safe for ingestion by most cow's milk-allergic infants.

A few other foods, such as peanut oil, could be characterized as hypoallergenic because they lack the allergens from the source foods. However, these products are not marketed in this manner, and it might be unwise to take this approach unless each preparation is verified to be reproducibly free of allergens.

IV. DOSE-RESPONSE RELATIONSHIPS

A. Sensitivity

Although the level of exposure to a specific protein necessary to sensitize an individual is unknown, individuals with preexisting IgE-mediated food allergies can respond adversely to extremely low levels of the offending food. The interaction of food allergens with the IgE antibodies on the surface of the mast cell or basophil membrane leads to the release of comparatively large quantities of biologically active mediators of the allergic response including histamine and the leukotrienes. A small amount of allergen interacting with IgE may elicit clinically significant adverse reactions. The lowest dose of a food allergen that is likely to elicit an adverse reaction cannot be calculated with any degree of certainty for all individuals. This is because the tolerance for an offending food differs from one food to another and one individual to another.

The best estimates of the lowest tolerated dose in most individuals for various allergenic foods can probably be derived, however, from carefully taken histories of actual allergic reactions and extrapolations based on experiments with carefully controlled double-blind food challenges. Anecdotal reports indicate reactions from such incidental contacts as opening packages of the offending food,¹³⁹ wiping counters or tables containing peanut residues,¹⁴⁰ inhaling vapors of the cooking food,¹⁴¹ and kissing the lips of someone who has eaten the offending food.¹⁴¹ Occasionally, severe reactions can occur from contamination with trace amounts of the offending food. For example, the ingestion of french fries that were fried in the same oil used for the deep-fat frying of fish likely caused a fatal reaction in a fish-allergic patient.¹⁴² Exclusively breast-fed infants have also developed food allergies to foods such as peanuts ostensibly through exposure to food allergens in the breast milk as a result of ingestion of the offending food by nursing mothers.¹⁴³⁻¹⁴⁵ The extent of exposure to the allergenic food proteins via breast milk must be quite small. However, in most of these cases, the infants become sensitized, but do not react until exposure to a larger quantity of the allergen during the weaning period.¹⁴⁴ Although these experiences and

anecdotes confirm that very small amounts of food allergens will provoke allergic responses in some sensitive individuals, they do not provide quantitative estimates of the lowest tolerated dose. The severity of the allergic reactions is also likely to be directly related to the dose of exposure. Therefore, exposure to very small amounts of an allergenic food is not likely to elicit severe reactions in most cases.

Quantitative estimates of the lowest tolerated dose can be derived from actual allergic reactions in patients from whom careful histories are taken regarding the amount of the offending food ingested. Settiple and Settiple¹⁴⁶ estimated that ingestion of 1 to 2 g of shrimp and 25 mg of peanut in allergic individuals will provoke adverse reactions. Gern et al.¹⁴⁷ investigated several cases of allergic reactions to milk that occurred with food products that did not declare the presence of milk on the label. Some of the cases resulted from ingestion of a frozen dessert made from soybeans that were probably contaminated from the use of equipment that was also used to process dairy products. Here, a single serving contained the equivalent of approximately 2.5 ml of cow's milk.¹⁴⁷ This amount of milk would provide about 80 to 100 mg of milk proteins, 80% of that being caseins and 20% being whey proteins. Several cases involved the ingestion of hot dogs that contained partially hydrolyzed casein as a natural flavoring. Each hot dog contained an amount of casein found in approximately 0.3 ml of cow's milk,¹⁴⁷ which would provide about 10 to 12 mg of milk proteins. Jones et al.¹⁴⁸ investigated an allergic reaction to milk from a raspberry sorbet product that had become contaminated with milk through the use of shared equipment in the processing plant. The milk-allergic child reacted to the ingestion of an unknown amount of the sorbet containing 0.52% milk protein.¹⁴⁸ Sunflower butter processed on equipment used to manufacture peanut butter was responsible for an allergic reaction in a peanut-allergic individual who ate less than 0.25 teaspoonful of sunflower butter contaminated with approximately 1% peanut.¹⁴⁹ Yman et al.¹⁵⁰ reported a series on incidents related to inadvertent or unexpected exposure to allergenic foods that resulted in allergic reactions among sensitive individuals. Many of these incidents involved exposure to small quantities of the

offending food including milk in nine incidents at levels ranging from 0.04 to 1.1%, egg in three incidents at levels ranging from 0.003 to 0.16%, wheat gluten in three incidents at levels ranging from 0.3 to 11.9%, soy protein in three incidents at levels ranging from 0.5 to 7.0%, and hazelnut in one incident at a level of 0.2%. Quantitative exposure information was available in several of these cases. Fatal anaphylaxis occurred in a milk-allergic consumer after eating approximately 100 g of a sausage product containing 0.06% undeclared casein that equated to 60 mg of casein.¹⁵⁰ An asthmatic reaction occurred in a hazelnut-allergic consumer following the ingestion of about 3 to 6 g of a chocolate confectionery product containing 0.2% of undeclared hazelnut that equated to 6 to 12 mg of hazelnut.¹⁵⁰

Perhaps the best estimates of the lowest tolerated dose can be obtained from DBPCFC. These challenges have been conducted for diagnostic purposes rather than for determining the lowest tolerated dose. The typical protocol involves starting at a dose that is one half or less than the amount of the offending food estimated by the patient to provoke symptoms.¹⁵¹ A typical starting dose is often 400 to 500 mg,¹⁵² although considerable physician judgement is always involved in the selection of the starting dose. In fact, some physicians choose not to perform blinded food challenges on patients with histories of life-threatening anaphylactic reactions.¹⁵² Therefore, these estimates of the lowest tolerated dose may exclude some of the most sensitive patients. Atkins et al.¹⁵³ demonstrated that cottonseed meal protein elicited adverse reactions in sensitive individuals at doses ranging from 100 to 350 mg. Sampson¹⁴⁰ indicated that 50 to 100 mg of peanut protein elicited adverse reactions in some allergic children in DBPCFC. In their pioneering studies on the use of rush immunotherapy to decrease the severity of peanut allergy, Oppenheimer et al.¹⁵⁴ elicited adverse reactions in peanut-allergic patients at doses as low as 30 mg before immunotherapy, although the provoking doses ranged from 30 mg to 8 g with an average of 4 g. In patients with histories of life-threatening symptoms, Oppenheimer et al.¹⁵⁴ initiated DBPCFC at 1 mg of defatted peanut. Other peanut-allergic patients were started at a dose of 100 mg of defatted pea-

nut. The upper limit in DBPCFC is typically 8 g of the offending food.¹⁵¹ In the studies of Oppenheimer et al.,¹⁵⁴ a cumulative dose of 15.8 to 15.9 g was reached, although the maximum single dose was 8 g. Certainly, many food-allergic individuals do not react until several grams of the offending food are ingested. These experiences demonstrate convincingly that very small doses of food allergens can elicit allergic reactions. Milligram quantities of allergenic foods have elicited allergic reactions in sensitive individuals. Although a precise estimate of the lowest tolerated dose cannot be ascertained reliably, considerable caution should be exercised to limit consumer exposure to known allergenic proteins.

B. Severity as a Function of Dose

As with all toxic responses, allergic reactions display a dose-response relationship and become more severe as a function of dose. This is clearly the case in dose-response relationships observed in intradermal skin testing (ST). The evidence supporting this statement with food allergies is mostly observational and anecdotal. Again, the experience from DBPCFC demonstrates that a threshold exists at a given time in each patient below which no clinically significant reaction is evident. As noted above, the thresholds are quite low for many patients. The DBPCFC is stopped at the lowest dose eliciting adverse reactions. Because human subjects are involved, it would be unethical to determine in any controlled way that a higher dose would provoke a more serious reaction. However, most patients experience rather mild reactions in DBPCFCs, and describe much more severe symptoms occurring during actual allergic reactions where more of the offending food was likely eaten.

C. Sources and Detection of Trace Exposures

The primary means of preventing food-allergic reactions is to follow a specific food avoidance diet.^{141,155} Because allergic individuals can react adversely to ingestion of trace quantities of

the offending food, the success of this preventive measure can be compromised by the presence of small amounts of one food contaminating another. Trace quantities of the offending food can be obtained from a number of personal practices, in foodservice facilities, and in processed foods facilities, although a number of these situations have only been described anecdotally.¹⁵⁶

At the personal level, trace elements may be obtained when attempting to remove the offending food from a mixture before eating; using shared serving utensils, containers, cooking vessels; touching utensils, containers, countertops; handling the offending food; kissing the lips of someone who has just eaten the offending food; opening packages of the offending food; inhaling vapors from cooking of the offending food; and transferring food allergens from mother to infant via breast milk. In foodservice facilities, exposure to the offending food occurs primarily because the foods are not labeled. Allergic individuals must rely on information provided by waitstaff and cooks, which can be erroneous. Creative formulation of restaurant foods, such as the use of peanut butter in chili, can also lead to unexpected exposure to an offending food.¹⁴² In such situations, allergic consumers can inadvertently ingest rather significant quantities of the offending foods. Foodservice facilities can also be sources of exposure to trace residues of the offending foods through shared use of serving utensils, containers, griddles, cooking pots; use of the same frying oil for more than one type of food; and inhalation of cooking vapors from tableside food preparation. Food-processing practices can also lead to the contamination of one food with another. In many of these situations, the labels of the packaged foods do not acknowledge such contamination. Common practices in the processed foods industry that can lead to such contamination include the use of shared processing equipment; use of rework (leftovers from one formulation added to another); presence of allergenic proteins in other ingredients such as natural flavors and starches; mistakes in formulation; use of the wrong packaging material with resultant inaccurate labeling; and switching ingredients without altering labels.

Although these situations may occur fairly frequently, only a few such incidents have been well described. The use of shared equipment has resulted in product contamination and allergic reactions in several situations.^{147,149,157} Switching natural flavoring ingredients in hot dogs from a yeast autolysate to a partially hydrolyzed casein product resulted in allergic reactions.¹⁴⁷ Porras et al.¹³⁷ detected soybean protein in several soy-based ingredients, including lecithin and oil. Yman et al.¹⁵⁰ have documented numerous incidents in Sweden involving the undeclared use of allergenic ingredients in packaged foods, the use of shared equipment in ice cream, pasta, and chocolate processing, the labeling of spelt pasta as wheat free (when spelt is actually a type of wheat), the changing of formulations without changing labels, and the presence of allergenic proteins in ingredients such as flavorings and binders. Occasionally, more bizarre forms of contamination can occur such as the contamination of beignet mix with *Dermatophagoides farinae* mites, which are highly allergenic when present in house dust.¹⁵⁸

Immunoassays can detect residues of allergenic food proteins in other foods.³³ These immunoassays, which can involve the use of serum from allergic individuals that contains allergen-specific IgE antibodies,^{137,147-149,159} are quite reliable. They detect the actual allergen, but are not commonly available because they rely on serum from allergic individuals. Animal antisera and monoclonal antibodies have also been used effectively in such analyses.^{150,160,161} These antibodies are not always directed specifically at the allergenic proteins, but can be quite specific for detection of proteins from a particular food.

V. TESTING FOR FOOD ALLERGENS AND THE ALLERGENICITY OF FOOD PROTEINS

The *in vitro* and *in vivo* tests described below, which use human subjects or samples, are only suitable to investigate allergenicity to already-sensitized individuals. These tests cannot be used to predict the sensitizing potential of novel proteins. Animal models, although not yet fully developed, offer the only future hope for improvement of such assessments.

A. *In Vitro* Tests

1. Immunoassays and Inhibition Immunoassays

The radioimmunoassay (RIA) and RIA inhibition procedures have been described in some detail by Yunginger and Adolphson.¹⁶² In the RIA (Figure 2), an extract of the food is attached to a suitable solid phase. The serum from individuals with allergies to that food is incubated with the solid phase allowing the allergen-specific IgE antibodies to bind to the solid-phase allergen. The bound IgE antibodies are then detected with the aid of ¹²⁵I-labeled anti-human IgE. In an alternative enzyme-linked immunosorbent assay (ELISA) procedure, enzyme-labeled anti-human IgE is used. These immunoassays rely on the availability of blood serum from food-allergic individuals. Unlike *in vivo* tests, these individuals do not have to be physically present during the test.

In the inhibition format, an extract of the food is bound to the solid phase just as in an immunoassay. The IgE-binding ability of a test extract (e.g., a processed form of the food) is assessed by mixing increasing concentrations of the test extract with human sera from food-allergic individuals and allowing the mixtures to incubate with the solid phase. A competition occurs for IgE binding to the solid phase vs. the test extract. The amount of IgE bound to the solid phase is determined using ¹²⁵I-labeled anti-human IgE. An inhibition curve is developed which plots the counts bound to the solid phase vs. the protein concentration in the free phase or test extract. The inhibition immunoassay has been used successfully to detect trace residues of allergenic proteins contaminating other foods,^{147-149,157,159} and to assess the effect of processing on the allergenicity of various products made from peanuts and soybeans.^{51,54}

2. Crossed Immunelectrophoresis and Radioimmunelectrophoresis

CIE and CRIE have been used to separate and detect the multiple allergens that exist in most foods.^{74,163,164} Although these procedures have

some limitations and have been largely supplanted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), these procedures could still be useful in the detection of specific allergens. The general procedures for CIE and CRIE were described in some detail by Yunginger and Adolphson.¹⁶² The major limitation by comparison to SDS-PAGE is the comparative lack of resolution of individual proteins in the agarose gels used in CIE and CRIE.

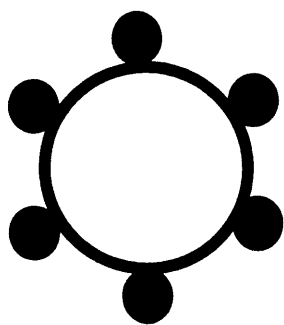
3. SDS-PAGE and Immunoblotting

The separation and detection of allergens is perhaps best accomplished by SDS-PAGE with immunoblotting. This procedure has been frequently applied to the identification of multiple allergens existing in many foods.^{29,43,50,60,61} However, the clinical significance of the individual IgE-binding proteins revealed by SDS-PAGE has not been determined, especially with regard to minor allergens (see "Food Allergens"). In SDS-PAGE, a food protein extract is treated with SDS to disrupt polymeric proteins into their simplest monomeric subunits.¹⁶⁵ These proteins are then separated by PAGE by a variety of slightly different procedures. Immunoblotting techniques are thoroughly described elsewhere.^{166,167} The separated proteins are electrotransferred to nitrocellulose; the nitrocellulose is overlaid and incubated with human sera containing IgE antibodies to the particular food extract; and the IgE-binding proteins are detected using either enzyme-labeled or radiolabeled anti-human IgE antibodies and a subsequent colorimetric enzymatic reaction or autoradiography as appropriate.

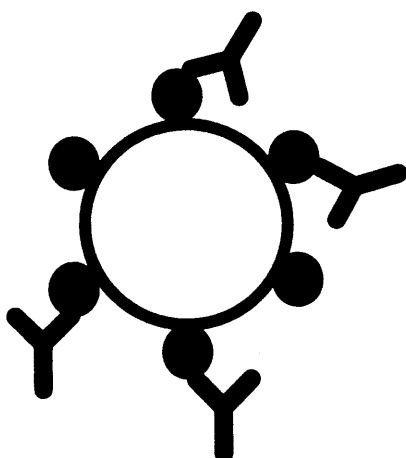
B. *In Vivo* Tests

1. Skin Tests

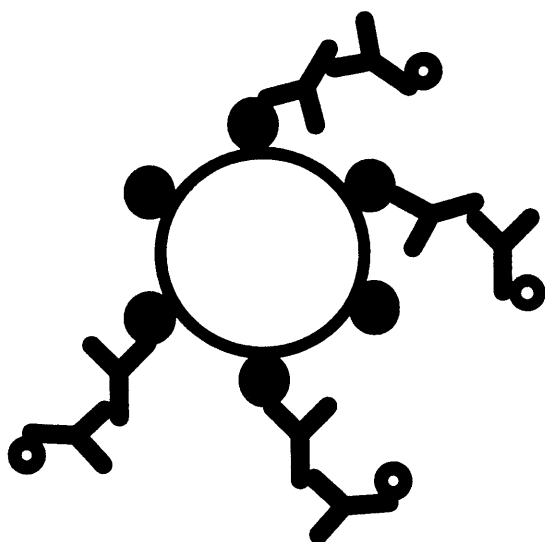
The skin prick test is probably the most frequently used test for the diagnosis of allergies including food allergies. It is typically conducted by the prick/puncture method with diluted (1:10 or 1:20) extracts of the specific food. A positive skin test is indicated by the development of a



Antigen attached to solid phase (eg. peanut protein attached to cyanogen bromide activated cellulose)



Antigen specific immunoglobulin binds to protein attached to solid phase



Bound IgE detected with labeled anti-human IgE

FIGURE 2. Radioimmunoassay (RIA) for food-specific IgE antibodies.

wheal 3 mm or larger in diameter at the puncture site than by a diluent control wheal measured within 10 to 30 min of antigen treatment. The

proper application of the skin prick test in the evaluation of food allergies was described by Bock et al.¹⁶⁸ When used in the evaluation of the

allergenicity of specific proteins, the skin test extract is typically prepared from purified or semipurified material. The standardization of allergen extracts would be desirable to enhance the reliability of skin tests (see "Allergic Reactions to Foods" by John A. Anderson in this issue).

2. Challenge Tests

The DBPCFC is considered to be the gold standard for the diagnosis of adverse reactions to foods.¹⁵¹ The DBPCFC is often used in the diagnosis of adverse reactions to foods (see "Allergic Reactions to Foods"). For purposes of testing the allergenicity of food proteins, a group of patients with well-documented histories of allergic reactions to the food under study, who also have positive skin tests or immunoassays, are selected. Patients with severe reactions would not participate in DBPCFC.

For the DBPCFC, the food must be disguised in some fashion. Usually, the food is dried in some way, and the dried product is encapsulated in opaque, tinted gelatin capsules.¹⁵¹ The amount of product that can be placed in one capsule usually ranges from 400 to 500 mg, although some exceptions may exist. Alternatively, the food may be given in a liquid vehicle such as VivonexTM or a milk shake; the food may also be disguised in another solid food such as a hamburger or apple sauce.¹⁵¹ The taste of the food must be thoroughly disguised in whatever vehicle is used. Often, capsules are wiped clean of any excess food and then dipped in powdered sugar to provide a uniform and sweet taste.¹⁵¹ In liquid or solid vehicles, the taste of the vehicle must effectively disguise any taste conveyed by the test substances. Strong flavors such as grape or chocolate for liquid vehicles are often preferred.

The DBPCFC is typically performed over a range of doses. The starting dose should provide no more than one half of the food that was present in the minimally provoking dose by history. The maximum dose should be 8 g of the dried food product in a capsule challenge, although larger quantities might be used with liquid or solid vehicles in adults.

A variety of placebos can be considered.¹⁵¹ Obviously, with liquid or solid vehicles, the placebo is the vehicle without the test substance. Placebo capsules are often filled with sugar or some other readily metabolized ingredient. DBPCFCs are strongly preferred over either open challenges or single-blind challenges. Open challenges should be considered as a follow-up to negative DBPCFC to assure that reactions would not occur under typical preparation practices.

VI. UTILIZATION OF TEST METHODS IN THE EVALUATION OF THE ALLERGENICITY OF GENETICALLY ENGINEERED FOODS

A. In Vitro Tests

1. Immunoassays and Inhibition Immunoassays

In immunoassays with genetically engineered foods, the newly introduced or expressed protein could be attached directly to the solid phase if available in purified form. Alternatively, an extract of the genetically engineered food could be attached to the solid phase. In this case, the amount of the newly introduced or expressed protein in the extract actually attached to the solid phase should be quantified. The extract must be prepared in a fashion to assure extraction of the newly introduced or expressed protein as discussed below for skin test extracts. Ideally, the concentration of the newly introduced or expressed protein attached to the solid phase should be equivalent to that found with a solid phase prepared with the donor food material.

The immunoassays can be more easily used in the detection of allergens from genetically engineered foods than the skin test (see Section VI.B.1) because the patients do not need to be assembled in one location. This is particularly advantageous for less commonly allergenic donor foods. Of course, the serum must be drawn from individuals with well-documented allergies to the donor food in question.

Although the immunoassays will be useful in the evaluation of the allergenicity of genetically

engineered foods, the inhibition immunoassays should be even more useful. In the inhibition immunoassay, a protein extract of the donor food material would be attached to the solid phase. An extract would be made of the genetically engineered food with all of the caveats as noted for skin test extracts (see Section VI.B.1). Increasing concentrations of this genetically engineered food extract would be mixed with human serum from allergic individuals with the appropriate allergy to the donor food and allowed to incubate with the solid phase. A competition would occur between the donor food allergen bound to the solid phase and any allergen from the genetically engineered food contained in the free phase. Again, the amount of IgE bound to the solid phase would be determined using ^{125}I -labeled anti-human IgE. An inhibition curve plotting percent counts bound to the solid phase vs. the protein concentration in the free phase would be prepared.

The inhibition immunoassay method was used successfully to identify the allergenicity of a methionine-rich Brazil nut protein that has been cloned into soybeans to enhance their nutritional value.¹⁶⁹

2. SDS-PAGE and Immunoblotting

Immunoblotting following SDS-PAGE could be easily adapted to the analysis of genetically engineered foods containing new proteins derived from known allergenic foods. In these experiments, the genetically engineered food can be compared to the food from the host plant and the food from the donor. A comparison of the IgE-binding proteins among these three foods would reveal if the genetically engineered food contains any allergens derived from the donor. SDS-PAGE with immunoblotting is quite sensitive especially when radiolabeled antibodies and autoradiography are employed. Thus, the allergens would likely be detected even if the amounts expressed in the genetically engineered food were rather small. This technique has been used to determine that a high-methionine protein from Brazil nuts, which was transferred into soybeans to correct the sulfur amino acid deficiency, was the most prominent IgE-binding protein in Brazil nuts.¹⁶⁹

B. In Vivo Tests

Certain ethical issues must be considered in the use of human subjects in the evaluation of the potential allergenicity of genetically engineered foods. Institutional Review Board (IRB) approval must be obtained.

1. Skin Tests

The skin test for IgE antibody reactivity could be useful in the assessment of the allergenicity of genetically engineered foods in situations where the donor material is derived from known allergenic foods. Any assessment would require the identification of a group of individuals with a well-documented allergy to the donor food material (see "Assessment of the Allergic Potential of Foods Derived From Genetically Engineered Crop Plants" by Dean D. Metcalfe et al. in this issue).

The preparation of the extract from the genetically engineered food for use in the skin prick test is critical. Fresh foods should be used for the preparation of the extract in most cases, although the comparison of fresh and processed foods might be useful on occasion. Extracts of the donor food and the nonengineered host food could be prepared and included for comparative purposes. Most food extracts for skin prick testing are prepared by extracting the pulverized fresh food in saline. Some foods are defatted first, but only if enough fat exists in the food to interfere with extraction. The saline extract should contain the soluble albumin and globulins from the particular food. If the novel protein is known to be insoluble in saline, then some alternative extraction procedure might be devised. In a few cases, it may be unnecessary and unwise to make an extract. Extracts prepared from fresh fruits, are notoriously unstable. In these situations, the preferred method would be to use a drop of the fresh fruit juice on the skin or to puncture the skin by inserting the needle through the fruit or other food after placing it on the surface of the skin. The presence of the novel protein in the skin test extract should be confirmed and quantitated. The extent of the dilution of the saline extract can be adjusted, as necessary, to achieve a proper concentration. If the

saline extract does not contain sufficient amounts of the novel protein, some concentration step should be considered. The stability of the novel protein in the saline extract should also be determined. If this protein is unstable then extracts should be freshly prepared before each test.

2. Challenge Tests

The DBPCFC would be applied in the evaluation of individual(s) who have a well-documented allergic reaction to the food that is the source of the inserted genetic material in genetically engineered foods (see "Assessment of the Allergenic Potential of Foods Derived From Genetically Engineered Crop Plants"). The documentation of the allergic status of the individuals in the test group should be based on significant clinical histories of adverse reactions and positive DBPCFC to the source food, as well as positive skin tests and immunoassays for specific IgE antibodies.

ST and immunoassays with extracts of the genetically engineered food should be conducted before the DBPCFC. If positive skin tests were obtained with food extracts, extra precautions would need to be exercised in the DBPCFC.

The food used for DBPCFC challenge should be harvested, cleaned, and processed in a manner that would be typical for that food. The challenge food should contain as many of the components of that food as might be usually expected. For example, if evaluating a wheat, the wheat grains might be converted into a whole-wheat flour. The form of the product should be that suspected of causing a reaction. With oilseeds, the meal and the oil might be tested separately because the oiliness of whole peanuts could create difficulties in some administration vehicles. Although most foods would be dried for convenience before administration, fresh foods might be needed for some DBPCFCs. For example, allergic reactions to fruits usually only occur to fresh fruits so that fresh fruit would be the most appropriate test substance in that case. The dosage recommendations would be similar to those suggested for any DBPCFC. Alternatively, the purified recombinant protein could be used in DBPCFC, although IRB approval for human challenges might prove difficult.

When conducting DBPCFCs of genetically engineered foods, the placebo would be the food from the nonengineered variety. This placebo should add value to these tests because it would be analogous in taste, texture, and composition to the genetically modified food, except for the newly introduced proteins.

C. Animal Models

New animal models could also be evaluated for their potential to assess the allergenicity of foods derived from genetically modeled plants. There are several criteria that, in theory, such animal models of food allergy must satisfy. First, the test animal must tolerate the majority of food proteins to which it is naturally exposed while mounting a significant IgE antibody response to only a few allergenic ones. Second, exposure and challenge with an allergen must occur orally. Third, this model must reflect organ sensitivities similar to those seen in humans during a food-induced allergic reaction such as GI reactivity, dermatologic, and/or respiratory hypersensitivity. Finally, such a model must be relatively easy to induce and consistently reproducible, from one time interval to another within the same species and different laboratories. Unfortunately, such an animal model is not available now, and may not be in the near future. The only alternatives are animals that produce allergen-specific IgE antibodies in response to parenteral allergen immunization plus adjuvant.¹⁷⁰ Although these models are useful in food allergy research and allergenicity determinations, they are not adequate food allergy models.

Unfortunately, at this time there are no animal models that can accurately predict the allergenicity of food proteins or replicate a food-induced allergic response. The major problem is that the requirements for such a model are inherently strict and it is difficult to answer complex questions with only animal models. Another difficulty with animal models is that all IgE antibody responses are not the same. Mice and rats differ in IgE antibody responsiveness,¹⁷¹ and even different strains of mice can differ from one another.^{172,173} This, however, may not necessarily be a deficiency, because it does reflect the variety of

IgE responsiveness seen in humans. Another problem is that the responsiveness of experimental animals does not always remain the same. Kemeny¹⁷¹ has reported that changes in an animal's IgE responsiveness were observed from 1 year to the next in his own laboratory under conditions using identical allergen and immunization methods.

It is very difficult to get significant IgE antibody responses in animals such as mice (the best studied) without the use of adjuvants such as aluminum hydroxide¹⁷² or *Bordetella pertussis*.¹⁷³ Perhaps the best investigated is the *B. pertussis* vaccine; yet the component inducing this response has not been fully characterized. More recently, Kemeny used the castor toxin, ricin, to induce IgE antibody responses. Using this substance he demonstrated that CD8⁺ T cells were involved in the regulation of IgE antibody responses.¹⁷⁴

A final consideration for animal models is that animals must respond to ingested or gavaged allergens. Unfortunately, there is very little information on responses of animals exposed to allergens in this fashion, particularly in the absence of adjuvants. Over the years a number of investigations have used animals to study food-induced allergic responses. Jarrett¹⁷⁵ studied rats responding to antigens administered by mouth or by injection, and reported that by either route, antigen diminished rather than heightened IgE responses to a subsequent allergen exposure. These animals have a highly developed capacity to suppress the production of IgE by immunoregulatory mechanisms. An interesting intestinal anaphylaxis model in rats was developed by Byars and Ferraresi.¹⁷⁶ Changes in permeability of the intestine after oral challenges of sensitized rats was the parameter measured through uptake of ¹²⁵I-labeled BSA. Other models used intestinal anaphylactic reactions in sensitized mice¹⁷⁷ and a rabbit hyperimmunization model¹⁷⁸ to assess the potentially reduced allergenicity of treated, compared with untreated cow's milk. Although the authors claim some promising results from these models, this is in contrast to what has been reported for assessment of other hypoallergenic formulas.

Bozelka and colleagues¹⁷⁹ attempted to develop a food allergen model in C₃H/HeJ mice immunized parentally or intragastrically with two food allergens, soy and shrimp, without signifi-

cant success. Although both preparations elicited specific IgE antibody responses to allergen when given intraperitoneally, soy or shrimp administered intragastrically with or without pertussis adjuvant did not induce detectable IgE antibody responses.

There is no adequate animal model to measure food allergens in terms of a full range of their activities. There may be some hope for an animal model to assess a protein's ability to induce IgE antibody response, but this requires further research. One needs to be careful about over reliance on any animal model in terms of its ability to predict the allergenic potential of a particular food substance in humans. An unfortunate example of this is efforts to develop hypoallergenic infant formulas based on a partial hydrolysis of whey.⁷⁶ These products were screened for immunogenicity in rabbit animal models measuring IgG antibody responses. These models predicted decreased immunogenic activity, and the products were marketed as hypoallergenic infant formula. In reality, these partial whey hydrolysates still contained substantial allergenic activity that triggered allergic reactions when fed to cow's milk-allergic infants.^{79,80,126} One must view with caution interpretation of any studies based solely on such animal models.

VII. SUMMARY

Food allergens are typically proteins that are resistant to digestion, acid, and heat treatments. However, some notable exceptions exist. The amount of these proteins needed to elicit an allergic response in previously sensitized individuals is quite low (milligram amounts). The allergenicity of specific proteins derived from known allergenic sources can be determined, whereas the potential allergenicity of proteins derived from sources of unknown allergenicity is much harder to assess.

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