

FORUM

Why Are Some Proteins Allergens?

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The ability of certain proteins to induce an allergic response in susceptible individuals is well established. Symptoms can range from mild erythema or rhinitis, to acute, and possibly fatal, anaphylactic shock. Because such allergic responses require complex interactions between the protein and the immune system, they are notoriously difficult to predict. Nevertheless, it is clear that some proteins are intrinsically more allergenic than others. The challenge for toxicologists is to identify those characteristics that confer on proteins the potential to induce allergic sensitization and allergic disease. Here, we first consider the potential contribution that individual epitopes may make to the allergenicity of a protein. These are the minimal peptide units within proteins that can be recognized by the immune system and are a fundamental requirement for all immune responses, including those resulting in allergic sensitization. It appears that allergens must necessarily contain B-cell epitopes to which immunoglobulin E (IgE) can bind, and T-cell epitopes capable of inducing a type 2 T-lymphocyte response. Nevertheless, it appears doubtful that the presence of appropriate epitopes alone is sufficient to endow a protein with allergenic potential. We therefore consider also the contribution that other features and characteristics of proteins may make to their overall allergenicity. In particular, we consider the effects that resistance to proteolysis, post-translational glycosylation, and enzymatic activity may have. It appears that relative stability in simulated gastric fluid (SGF) sometimes correlates with allergenic activity. However, this is not universally true, and it is known that there are protein allergens, such as some of those associated with oral allergy syndrome, that are unstable. Nevertheless, if stability in SGF is associated with the intrinsic allergenicity of many proteins irrespective of the route of exposure, then this may reflect some more fundamental property of proteins, and possibly their stability in other biologic matrices and/or to intracellular proteases. Post-translational modification appears generally to enhance allergenicity, perhaps by increasing uptake and detection of the protein by the immune system. Some enzymatic activities also enhance allergenicity through what appear to be several different mechanisms, including nonspecific activation of cells participating in the immunologic response. Overall, it appears likely that many

factors can contribute to the overall allergenicity of any given protein. Some, such as the presence of epitopes with allergenic potential, may be essential. Others, such as the glycosylation status, resistance to proteolysis, and enzymatic activity, may play a subsidiary but nevertheless critically important role. By better defining the limits within which these factors operate, we can hope to gain a better understanding of the fundamental origins of protein allergenicity, and therefore be in a position to identify and characterize the hazards and risks of allergic disease associated with novel proteins.

Key Words: allergenicity; epitopes; proteins; simulated gastric fluid (SGF).

Introduction

In a toxicologic context, allergy is best defined as the adverse health effects that may result from the stimulation by a xenobiotic of a specific immune response. Allergic disease resulting from exposure to chemicals or drugs has been of interest to toxicologists for many decades. However, allergy to proteins (within foods and pollen, for instance) is more prevalent and has recently assumed greater significance for toxicologists with the increasing interest in the use of proteins as effect molecules. One example is the introduction of enzymes into detergents, as for some time it has been known that they have the potential to cause allergic sensitization (Flindt, 1969; Merget *et al.*, 1993; Pepys *et al.*, 1969; Pham and Mire, 1978; Weill *et al.*, 1971). More recently, attention has turned to the safety assessment of genetically modified crops that contain novel proteins. Here, a major challenge for toxicologists is to consider the possible allergenic properties of proposed transgenic proteins.

The most direct approach for determining the potential of proteins to cause allergy is to test the characteristics of the immune response they induce in animals. Variations on this theme include a guinea pig intradermal sensitization method (Arakawa *et al.*, 1995), a guinea pig intratracheal test (Ritz *et al.*, 1993; Sarlo *et al.*, 1997), a mouse intranasal test (Robinson *et al.*, 1996; Robinson *et al.*, 1998), oral dosing of rats (Atkinson *et al.*, 1996; Knippels *et al.*, 1999; Knippels *et al.*, 1998) or

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mice (van Halteren *et al.*, 1997a; van Halteren *et al.*, 1997b), and intraperitoneal injection of mice (Hilton *et al.*, 1994; Hilton *et al.*, 1997). There are, however, considerable variations between different approaches, highlighting a need for the development of robust, well-validated, and widely accepted methods that will facilitate the accurate identification of potential human protein allergens and assessment of sensitization risks.

A systematic, tiered approach to evaluation of the potential allergenicity of proteins introduced into crops was proposed in 1996 by Metcalf *et al.* (Kimber *et al.*, 1997; Metcalfe *et al.*, 1996; Taylor and Lehrer, 1996). For proteins derived from sources associated with allergy, it is recommended that immune reactivity with sera derived from a minimum of 14 subjects known to be sensitive to the food source be assessed *in vitro*. If all fail to react to the protein, it is considered unlikely that the protein is a significant food allergen. If equivocal results are obtained, it is proposed that skin prick tests on at least 14 individual allergic subjects should be performed. Where fewer than 5 subjects or their sera are available, *in vitro* analysis of protein stability in simulated gastric fluid (SGF) is recommended, based on the empirically observed correlation between stability and allergenic potential among proteins tested to date (Astwood *et al.*, 1996). Finally, double-blind placebo-controlled food challenges of subjects known to be sensitized to the food source are recommended as the best currently available test.

For proteins derived from sources considered not to cause allergy in humans, it is proposed that the amino acid sequence be compared with those of known allergens. If regions of homology with known protein allergens spanning eight or more consecutive amino acid residues are found, indicating the possible existence of common epitopes, then further testing for proteins from a known allergenic source is indicated. If no significant homology with known allergens is observed, the protein avoids immediate flagging as a likely allergen, although it is still recommended that stability in SGF be tested; stability despite a lack of homology would arouse a low level of concern as to potential allergenicity.

This approach may prove to be successful in assisting with the assessment of the allergenic potential of novel proteins. Nevertheless, a more detailed understanding of the mechanisms underlying allergenicity is necessary to provide greater confidence in the accuracy of such approaches, and also to advance the development of new tests on a more rational basis.

Allergy as an Immune Phenomenon

The intrinsic potential of a protein allergen to induce sensitization will be manifest only in susceptible individuals (with, among other factors, age, and heritable and acquired predisposition influencing susceptibility), and then only if the allergen is encountered in sufficient quantities and via a relevant route

of exposure. Thus, even sources of very potent protein allergens such as peanuts will cause sensitization in only a small fraction of the exposed population. Interindividual differences in susceptibility to allergic disease are complex and determined by both heritable and environmental factors. Further consideration of the polymorphisms in response to protein allergens are beyond the scope of this article, which seeks instead to consider the features and characteristics that confer on proteins intrinsic allergenic potential and which, in effect, distinguish between protein allergens and protein immunogens. Detailed accounts of the genetic and environmental influences that are believed to predispose towards atopy and allergic disease are available elsewhere (Bleecker, 1998; Chatila, 1998; Hopkin, 1990; Marsh, 1999; Rosenwasser, 1998).

The Elicitation Phase of an Allergic Reaction

Using the broad definition stated above, allergy describes the adverse health effects that result from the stimulation of a specific immune response. As such, allergic disease may result from immune responses of various types, e.g., allergic contact dermatitis results from cell-mediated immunity and the elicitation in the skin of a delayed-type hypersensitivity reaction. Other forms of allergic disease are associated with IgG antibodies and the formation of local or circulating immune complexes. In the context of food allergy and sensitization to ingested proteins, it is immunoglobulin E (IgE) antibody responses that are of greatest relevance in the vast majority of instances. Before considering the basis of IgE antibody-mediated allergic sensitization, it is necessary to distinguish between true food allergy, which by definition is associated with and dependent upon the elicitation of a specific immune response, and nonimmunologic food intolerance, which is due to one or more of a variety of factors.

Most foreign proteins, except for those that are extensively conserved, are immunogenic. Some (probably a minority) are able to induce the quality of immune response that results in immediate-type allergic reactions. In this context, this equates with the ability of protein allergens to provoke IgE antibody responses. In common with antibodies of all isotypes, IgE can bind specifically via its F(ab) arms to the protein antigen that induced its production. Antibodies of the IgE isotype, however, are also able to bind via their constant (Fc) regions to specific IgE receptors (FcεR) found on the surface of (among other cells) mast cells and basophils. When two or more of these captive IgE molecules bind to their specific antigen, the FcεR can become cross-linked on the surface of the cell, initiating intracellular signaling events (Costello *et al.*, 1996). These signals activate the cells, leading to degranulation with the release of histamine, and the production of prostaglandins, leukotrienes, and other inflammatory mediators (Fig. 1) (Metzger, 1999; Ravetch and Kinet, 1991). Together, these agents mediate the symptoms of immediate-type hypersensi-

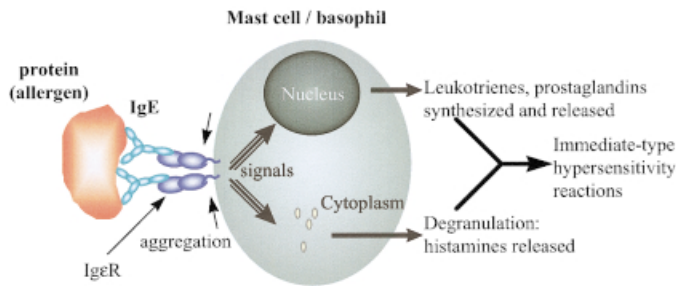


FIG. 1. Immediate-type hypersensitivity is mediated by factors released by mast cells and basophils as a response to intracellular signals generated by the IgE ϵ R on the surface of such cells. These signals result when multiple IgE molecules, associated with the IgE ϵ R molecules, bind with high affinity to a protein allergen. Thus, immediate-type hypersensitivity is dependent upon the presence of multiple IgE binding sites (two sites shown here for example) on the surface of a protein allergen.

tivity reactions. Particular subclasses of IgE may be induced preferentially by allergens, perhaps lending further specificity to this process (Inganäs *et al.*, 1980; Peng *et al.*, 1997; Peng *et al.*, 1994).

The cross-linking of Fc ϵ R requires that at least two antibody molecules bind to the inducing allergen. An allergen must therefore contain at least two IgE binding sites (epitopes), each of which will be a minimum of approximately 15 amino acid residues long, in order that antibody binding can occur. This implies a lower size limit for protein allergens of approximately 30 amino acid residues (M.W. of approximately 3 kD). In reality, it appears that most allergens have more than two IgE binding sites; the peanut allergens Ara h 1 (Shin *et al.*, 1998) and Ara h 3 (Rabjohn *et al.*, 1999), for example, are known to have at least 23 and 4 linear epitopes, respectively, whereas the major soybean allergen Gly m Bd has 16 (Helm *et al.*, 1998). Further discontinuous epitopes, undetectable in screens with synthetic linear peptides, may also exist in these proteins. In the case of Ara h 1, it has been found that IgE epitopes are arranged in two clusters on the 3-dimensional surface of the molecule, which may result in increased efficiency of cross-linking of IgE associated with the Fc ϵ R on the surface of mast cells (Shin *et al.*, 1998).

As reviewed recently (Singh *et al.*, 1999), some investigators have sought to lower the allergenic potential of proteins by altering their IgE-binding B epitopes. In this way, the ability of existing IgE to bind to the altered protein, and hence activate mast cells, can be reduced (Ferreira *et al.*, 1998; Ferreira *et al.*, 1996). As an adjunct to immunotherapy, where sensitized individuals are treated with tolerogenic doses of an (altered) allergen, this approach shows some promise. However, this aims only to reduce the severity of reactions in already sensitized subjects. To understand the underlying mechanism of allergic sensitization, it is necessary to consider events that occur when naïve individuals encounter allergens for the first time.

The Induction of Allergic Sensitization

Allergic sensitization to proteins involves the induction of an IgE response of sufficient magnitude to facilitate the elicitation of an inflammatory reaction following subsequent exposure to the same (or a cross-reactive) allergen. Sensitization occurs when these IgE antibodies are distributed systemically and bind to Fc ϵ R on mast cells and basophils. This raises the question, Why does only this minority of proteins elicit such an IgE response? To address this, it is necessary to consider the events that occur during the first encounter of an individual with protein allergen, and that may result in allergic sensitization (Fig. 2).

For a B cell to differentiate into a plasma cell and produce antibodies, the clonotypic B-cell receptor expressed on its surface must bind to specific B epitopes on the surface of the protein antigen. The antibodies subsequently produced by the plasma cell, into which the B cell differentiates, have the same specificity as the B-cell receptor, and are therefore able to bind specifically to the same B epitopes on the surface of the protein antigen.

Efficient secretion of antibodies normally requires that the B cells receive help from T-helper (Th) cells that specifically recognize separate epitopes on the same protein antigen. Such recognition is mediated by the clonotypic T-cell antigen receptor, which delivers stimulatory intracellular signals to the T cell. These signals are able to activate resting, immature Th cells (designated Th0), causing their differentiation into mature, effector Th cells, which can be of either type 1 (Th1) or

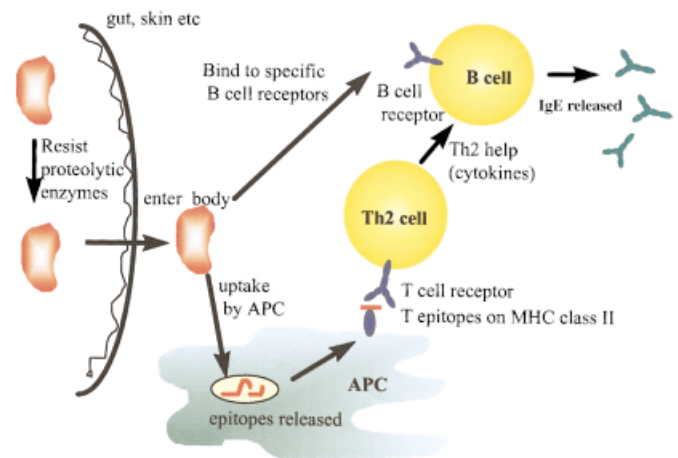


FIG. 2. In order for immunoglobulin (Ig) antibodies to be released from a B cell, two signals must be delivered: first, the clonotypic B-cell receptor must bind specifically to B epitopes on the surface of the antigen. Second, help from T cells in the form of cytokines must be delivered by specific T cells. For B cells to respond by releasing IgE, this help must be predominantly of type 2, i.e., provided by Th2 cells. For Th2 cells to be activated, their clonotypic receptors must recognize fragments of the antigen in question (T epitopes) that have been processed by APCs and displayed on their surface in association with class II molecules. Thus, the generation of IgE by B cells is controlled by Th cells responding to epitopic sequences within the antigen (see text).

type 2 (Th2). These mature T cells can help B cells to secrete immunoglobulin in two ways. First, they can stimulate accessory molecules on the B-cell surface, an effect mediated by cell/cell contact. Second, they can release cytokines into the milieu that can bind to, and hence activate, specific cytokine receptors on the surface of the B cells (O'Rourke *et al.*, 1997; Takatsu, 1997). The identity of the cytokines that stimulate the B cell in this way can affect both the strength and the quality of the antibody response. Typically, Th2 cells produce a cocktail of cytokines including interleukin (IL) -4, IL-5, IL-6, IL-10, and IL-13 that, among other actions, encourage plasma cells to switch to synthesis of IgE (Del Prete *et al.*, 1986; Del Prete *et al.*, 1988; Maggi *et al.*, 1987; Maggi *et al.*, 1988; Romagnani *et al.*, 1989a; Romagnani *et al.*, 1989b; Romagnani *et al.*, 1991). Conversely, Th1 cells typically produce IL-2, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α/β , that together downregulate IgE synthesis. Once the polarity of the Th response is established, it tends to be maintained by the suppressive effects of Th1 cytokines on Th2 cell development, and vice versa (Fernandez-Botran *et al.*, 1988), and usually cannot be reversed (Hansen *et al.*, 1999). The conjecture that Th2 cells contribute to allergenicity is supported by the finding that allergen-specific Th cells from allergic patients generally have a type 2 phenotype (Parronchi *et al.*, 1992; Parronchi *et al.*, 1991; Wierenga *et al.*, 1990). It is therefore intriguing to speculate how protein allergens may encourage the development of selective Th2 immune responses that in turn support the production of IgE antibody (Aebischer and Stadler, 1996; Comoy *et al.*, 1999; Del Prete, 1992; Maggi, 1998; Romagnani, 1998).

A Central Role for T Epitopes in Allergenicity

Allergenic proteins per se cannot be recognized by T cells; the proteins must first be processed and then presented by specialized antigen-presenting cells (APCs). These are located at potential points of entry into the body such as the gut, skin, and lungs, and can capture and internalize protein antigens from the surrounding milieu. Proteins are then partially digested within specialized proteolytic vesicles to generate sets of nested peptides. A fraction of these peptides bind with high affinity to major histocompatibility complex (MHC) class II determinants, which in immature APCs are associated with the vesicle membrane (Bartnes *et al.*, 1997; Reizis *et al.*, 1998). As APCs mature, they migrate to regional lymph nodes, and the assembled MHC class II/peptide complexes associated with the proteolytic vesicles are transported to the cell surface. The primary function of MHC class II is therefore to display peptides, derived from internalized antigenic proteins, on the surface of APCs. It is these complexes that can be recognized, dependent upon the identity of the peptide, by T cells. Specificity for a particular peptide is conferred upon a T cell by the T-cell antigen receptor, which is transcribed from a somatically rearranged antigen receptor locus, and is hence unique to each

T-cell clone (Barber and Parham, 1994). Only those T cells that by chance bear an antigen receptor specific for the peptide in question partake in the immune reaction against that peptide. Thus, from the perspective of T cells, proteins may be considered to be collections of discrete, immunologically recognizable epitopes embedded within the structural framework of a protein backbone.

It follows that an allergenic protein will contain T epitopes with the potential to induce Th2-type responses. Indeed, structural investigations of several allergens have identified T epitopes implicated in the selective development of Th2-type lymphocytes. Some allergens, such as ovalbumin, have only a few epitopes that preferentially induce a Th2 response, whereas others, such as β -lactoglobulin, have many (Sélo *et al.*, 1998). What remains unclear, however, is whether the potential to induce a polarized (Th1 or Th2) response is an intrinsic property of particular epitopes, or whether such potential is conferred upon epitopes by other characteristics of the protein allergen or the immune system with which it is interacting. This question is of practical as well as academic interest. Theoretically, the answer impacts our understanding of the causes of allergenicity. Practically, it may influence the selection of strategies that may be employed to minimize the allergenicity of proteins. If allergenicity is conferred by key epitopes, their modification would alter the allergenicity of a protein. If not, this strategy would fail to reduce allergenicity.

Allergenicity Inherent to T Epitopes

Many allergens share some homology, suggesting that allergenicity may, at least in part, be associated with primary sequence. Identifying such sequences would be of great value for predicting the likely allergenicity of untested proteins from their primary sequences, for deriving structure-activity relationships, and for exploring the molecular basis of allergy. However, when internal peptide sequences the length of T epitopes (10–15 amino acid residues long) are considered, no general homology across allergen families emerges. To date, it has proven impossible to identify a consensus sequence for an allergenic epitope (Gendel, 1998a; Gendel, 1998b). This cannot be interpreted as indicating that individual epitopes necessarily make only a small contribution to the overall allergenicity of a protein; it may rather reflect the existence of structural diversity among allergenic T epitopes for which no overall consensus need necessarily exist.

To examine whether T epitopes have an intrinsic ability to polarize the T-cell response they provoke, one approach has been to alter amino acid residues within defined T epitopes, and determine how subsequent immune responses to these altered peptide ligands are affected. A wealth of evidence suggests that altered T-cell ligands can alter the polarity of the T-cell response, both *in vitro* and *in vivo* (Nicholson *et al.*, 1998; Pingel *et al.*, 1999; Sloan-Lancaster and Allen, 1996; Tsitoura *et al.*, 1996; Vidal *et al.*, 1996; Windhagen *et al.*, 1995).

Mechanisms for Polarization of the Immune Response by T-cell Epitopes

Two potential mechanisms could account for these observations: either altered ligands could stimulate different clones of T lymphocytes with different intrinsic immunologic characteristics, or they could stimulate the same immature clones, leading to maturation into functionally discrete differentiated effector populations. There is little evidence for the first mechanism, although it has been shown that altered ligands can differentially engage mature Th1 and Th2 clones (Das *et al.*, 1997). Rather more attention has been focused on the second possibility, as there have been many demonstrations that individual T-cell clones may respond differently to altered T-epitope ligands compared with their wild-type counterparts (Chen *et al.*, 1996; Faith *et al.*, 1999; Sloan-Lancaster and Allen, 1996; van Bergen and Koning, 1998; Windhagen *et al.*, 1995).

Further investigations have revealed that the quality of the T-cell response to any given epitope depends upon many factors, including the dose of protein or peptide given, the affinity of the peptide for MHC class II, and the longevity of the class II/peptide complex (Kumar *et al.*, 1995; Murray *et al.*, 1994; Pfeiffer *et al.*, 1995; Pingel *et al.*, 1999). Together, these parameters act to influence the dynamic epitope density on the surface of APCs. It is generally agreed that parameters that serve to increase ligand density, including greater affinity for MHC class II (Kumar *et al.*, 1995; Murray *et al.*, 1994; Pfeiffer *et al.*, 1995; Rogers and Croft, 1999), higher levels of ligand loading (Constant *et al.*, 1995; Rogers and Croft, 1999; Schountz *et al.*, 1996), or longer-lived complexes (Rogers and Croft, 1999), all favor Th1-type responses.

This bias can be traced to the ability of high-density ligands to activate T cells more strongly than low-density ligands (Tao *et al.*, 1997a; Tao *et al.*, 1997b). Higher ligand density favors the cross-linking of T-cell receptor molecules (Viola and Lanzavecchia, 1996), and this can induce stronger intracellular signaling (Rabinowitz *et al.*, 1996; Smyth *et al.*, 1998). Strong intracellular signals can drive immature T cells to undergo more rounds of proliferation than weak ones. This in turn favors the establishment of a Th1-type response, as only undifferentiated T lymphocytes induced to undergo more than five rounds of division reliably differentiate into Th1 cells (Gudmundsdottir *et al.*, 1999). Surprisingly, this implies that the dominant epitopes of allergens should be displayed at relatively low density, or relatively transiently, in order to favor limited T-cell receptor cross-linking, fewer rounds of cell division, and polarization towards a Th2-type response. It must be emphasized, however, that antigen dose may affect more than the density of epitopes displayed to T cells; antigen doses can be altered experimentally such as to modulate IgE production without measurably affecting cytokine production (Arps *et al.*, 1998).

The potential of a protein to initiate an allergic response lies ultimately with epitopes that are recognized by the immune

system. This does not imply that the remainder of the protein that is not recognized specifically by T or B cells is irrelevant. The whole protein has a pivotal role in determining how and when constituent epitopes are presented to the immune system, thereby influencing the nature and vigor of subsequent immune responses. The remainder of this article will consider how the release and recognition of epitopes is affected by the architecture of the immune systems and by the structural characteristics of proteins.

Global Protein Characteristics

A survey of the common protein allergens reveals that they possess a wide range of physical characteristics, none of which is unique to protein allergens as a class. Nevertheless, some characteristics are more common among proven allergens than other proteins. One survey has suggested that allergens tend to be ovoid in shape, although it is unclear why this should contribute to allergenicity (Rouvinen *et al.*, 1999). Others have implicated repetitive motifs in allergenicity (Pomés *et al.*, 1998). Many of the important allergens are exceptionally heat stable and retain their allergenicity after heating (Taylor and Lehrer, 1996). However, proteins not considered to be allergens can be identified with any of the properties listed above, calling into question the predictive value of the observed correlations (Pomés *et al.*, 1998; Rouvinen *et al.*, 1999; Taylor and Lehrer, 1996).

Particular claims have been made regarding the contribution of intramolecular disulfide bonds to the allergenicity of proteins. Preventing disulfide bonding through site-directed mutagenesis has been found in the case of the allergenic proteins Der p 1 (Smith and Chapman, 1996) and Lep d 2 (Olsson *et al.*, 1998), both from house dust mites, to yield proteins that are no longer able to bind IgE derived from allergic patients. Although these studies showed that allergens may lose their immunologic identities (i.e., the ability to bind pre-existing specific IgE antibody) through the loss of disulfide bonds, they did not demonstrate whether the ability of altered proteins to initiate *de novo* IgE production, i.e., their intrinsic allergenicity, could be reduced by this means. This was considered by Buchanan *et al.* (Buchanan *et al.*, 1997), who showed that the induction of IgE antibody by wheat agglutinin could be lessened by reducing disulfide bonds with thioredoxin. They did not, however, determine whether disulfide bonds make a specific, qualitative contribution to the allergenicity of wheat agglutinin, or simply make a quantitative contribution to overall antigenicity. A quantitative reduction of the antigenicity of a protein could result in a reduction of the allergic response to a protein without altering its intrinsic allergenicity, if the vigor of the immune response is reduced. To determine whether the intrinsic allergenicity of a protein has been altered, it is necessary to assess independently its allergenicity and its antigenicity. This may be achieved experimentally by simultaneously measuring both the IgE (allergenic) and IgG (antigenic) re-

sponse to exposure. It nevertheless seems likely that disulfide bonds will influence allergenicity, though perhaps in unpredictable ways; their presence can profoundly affect the processing and stability of antigens, and hence the release or destruction of T-cell epitopes (Collins *et al.*, 1991). The presence of multiple intramolecular disulfide bonds per se does not make a protein an allergen, nor does their absence preclude allergenicity. For example, ovalbumin is an allergen despite having only a single intramolecular disulfide bond that contributes little to its stability (Takahashi and Hirose, 1992; Takahashi *et al.*, 1991), whereas bovine serum albumin (BSA), with 17 intramolecular disulfide bonds, is less allergenic (Hilton *et al.*, 1997).

Resistance to Proteolysis

A survey of the most common allergenic food proteins has found that most are relatively stable in SGF, whereas minimally allergenic control proteins are mostly labile (Astwood *et al.*, 1996). Because labile proteins are unlikely to persist in the gastrointestinal tract for sufficient time to provoke an immune response, it is predictable that they are typically nonallergenic when administered orally. Before considering this apparent relationship, it must be acknowledged that some proteins (frequently pollen, fruit, and vegetable proteins) are associated with what is known as the oral allergy syndrome (OAS) (Ortolani *et al.*, 1988), and many of these are recognized as being relatively unstable. Intriguingly, however, some correlation between allergenicity and stability in SGF appears to hold true even when animals are immunized via a non-oral route of exposure (Astwood *et al.*, 1996), in which case the stability of proteins in gastric fluid per se is irrelevant. Critically, most proteins, including those that are labile in SGF, are found to be antigenic (able to induce IgG) in mice that have been immunized via a non-oral route (Hilton *et al.*, 1994). However, only those proteins with significant allergenic potential provoke also an IgE antibody response under these exposure conditions. In these instances, the inability of SGF-labile proteins to initiate an allergic response cannot be accounted for by their failure to be recognized by the immune system in the gastrointestinal tract. This suggests that the stability of a protein in SGF may predict not only its allergenicity when administered orally, but also its inherent allergenicity, regardless of route of exposure. In this case, stability in SGF is presumably acting as a surrogate marker of other properties of proteins that contribute directly to their allergenicity.

One possibility is that stability in SGF reflects stability in other extracellular matrices, such as serum or lymph, that are encountered by proteins when administered by nongastric routes. It seems unlikely, however, that relative stability or lability of a protein in such biologic matrices could profoundly affect its allergenicity without a concomitant effect on its antigenicity.

Another possibility is that stability in SGF reflects stability in the acidic proteolytic vesicles of APCs wherein proteins are

processed and T-cell epitopes are released (Ramachandra *et al.*, 1999). This may lead to the differential processing of the proteins, which in turn can affect both antigenicity and allergenicity (Vijh *et al.*, 1998). For example, it is known that the dominant T-cell epitope of ovalbumin associated with allergenicity is generated following digestion by the aspartyl protease cathepsin D, but is destroyed by the cysteine proteases cathepsin B or cathepsin L (Diment, 1990; Rodriguez and Diment, 1995; Rodriguez and Diment, 1992). Ovalbumin is known to be directed preferentially to the mildly acidic MHC class II positive endocytic vesicles (Lutz *et al.*, 1997) characterized by an absence of the marker rab 7 (Bottger *et al.*, 1996; Press *et al.*, 1998). These vesicles are also known to be enriched in cathepsin D (Manoury *et al.*, 1998; Press *et al.*, 1998). The stability of ovalbumin is likely to be influenced critically by its relative exposure to these different proteases. It remains to be seen whether stability in SGF mirrors stability in intracellular proteolytic vesicles, or simulated intracellular proteolytic fluid, which would lend support to this hypothesis.

At this stage, relatively few proteins have been tested for both allergenicity and stability in SGF, so caution must be applied when attempting to extrapolate the results obtained so far into a general principle, particularly when underlying mechanisms are unknown. Rigorous testing of the hypothesis that stability in SGF is a predictor of allergenicity (without need for reference to the underlying mechanism) will necessarily involve the molecular manipulation of proteins to specifically alter their stability in SGF, and the concurrent assessment of their allergenicity. A first step in this direction has been taken by del Val *et al.* (1999), who found that the reduction of intramolecular disulfide bonds in lactoglobulin simultaneously increased sensitivity to peptic digestion and reduced its allergenicity, as assessed by skin prick testing in sensitized individuals. However, skin prick testing only assesses cross-reactivity between native and denatured lactoglobulin at the level of binding to pre-existing IgE in sensitized individuals; it does not assess directly the inherent allergenicity of the denatured protein, that is, its ability to initiate the production of IgE.

Glycosylation

Many protein allergens are glycosylated, raising the possibility that the glycosyl groups may contribute to their allergenicity. This is potentially of particular relevance when considering the allergenicity of transgenic proteins for which glycosylation patterns may differ substantially from their native counterparts (Jenkins *et al.*, 1996). Such differences are especially likely when transgenes are expressed at abnormally high levels, in tissues from which they are normally absent, or when wide species barriers are crossed. Surprisingly, any relevance of protein glycosylation to the induction of an allergic response to proteins has yet to be demonstrated definitively. Nevertheless, many potential mechanisms exist whereby sugar

groups could influence the immunogenicity and allergenicity of proteins, highlighting this as an area where further research is required.

Oligosaccharides are naturally added to many proteins during or shortly after their synthesis in eukaryotic cells. Glycosylation involves the covalent attachment of oligosaccharides most commonly to asparagine (N-linked) or serine/threonine (O-linked) amino acid residues, dependent upon the presence of flanking glycosylation motifs within the primary amino acid sequence (Marshall, 1972). As such, they are an integral part of the resultant glycoproteins, and influence their physical properties. These may include, among others, altered stability, solubility, hydrophobicity, and electrical charge. Any number of these changes may affect the stability and uptake of a protein, and hence alter its antigenic and allergenic potential. Glycosylation can also affect directly the immunologic properties of proteins in several ways, as considered below.

By altering the identity of residues present within the primary amino acid sequence, glycosylation can alter the B-cell epitopes present on the surface of a protein. It is found that glycosylated epitopes generally make very good B-cell epitopes (Petersen *et al.*, 1998; Weber *et al.*, 1987), with a significant proportion of the IgE directed against a glycoprotein binding to glycosylated epitopes (Batanero *et al.*, 1994; Singh *et al.*, 1999; Tretter *et al.*, 1993). Thus, the ability of a protein to elicit the clinical manifestation of an allergic response (i.e., cross-link IgE on the surface of mast cells) in a sensitized individual may be reduced by deglycosylation, as has been demonstrated, e.g., for the allergens Phl p 1 (Petersen *et al.*, 1998) and Ole e 1 (Batanero *et al.*, 1994). However, this may simply reflect a lack of identity between the initiating allergen (native, glycosylated protein) and the challenge protein (recombinant, nonglycosylated protein), which are likely to have only limited cross-reactivity. Critically, it remains to be determined whether, and to what extent, the glycosylation status of proteins influences their ability to induce IgE responses.

As for B epitopes, the identity of T epitopes may be altered through glycosylation, which may render them either more or less antigenic (Mouritsen *et al.*, 1994). Abnormally glycosylated peptides have been implicated in the induction of type II collagen-induced arthritis (Corthay *et al.*, 1998). Glycosylation of a protein may also affect the identity of nonglycosylated T-cell epitopes released during proteolysis by affecting the availability of proteolytic sites within the protein. It is noteworthy that asparagine residues, which may be potential glycosylation sites, are the specific targets of a protease shown to be involved in the initial digestion of proteins within APCs (Manoury *et al.*, 1998).

Glycosylated proteins and peptides can show several hundred-fold greater rates of uptake by cells than their nonglycosylated counterparts (Agnes *et al.*, 1998; Jansen *et al.*, 1991; Kindberg *et al.*, 1990). This holds true for the uptake of proteins by blood-derived dendritic cells, considered to be a model of natural APCs (Sallusto *et al.*, 1995). The cause of this

phenomenon can be traced to the presence of specific sugar receptors on the surface of these cells, such as the macrophage mannose receptor (MMR) (Condaminet *et al.*, 1998; Engering *et al.*, 1997), or the related receptor DEC 205 (Jiang *et al.*, 1995). Besides increasing the quantitative uptake of proteins, these receptors may influence the subsequent processing of the proteins by targeting them to distinct intracellular proteolytic compartments (Hewlett *et al.*, 1994; Lutz *et al.*, 1997). Thus, ovalbumin (a glycosylated allergen) and dextrin (a sugar that is pinocytosed) may be observed in discrete intracellular locations some hours after uptake (Lutz *et al.*, 1997). In that study it was assumed, but not proven, that the oligosaccharides on ovalbumin contribute to this differential uptake. In another investigation, ovalbumin, along with the mannose-6-phosphate receptor, was shown to enter a subset of endosomes distinguished by the absence of the marker rab 7 (Press *et al.*, 1998). These are enriched in the protease cathepsin D, which can release the dominant T-cell epitope of ovalbumin associated with allergenicity (Rhodes and Andersen, 1993; Rodriguez and Diment, 1995; Rodriguez and Diment, 1992).

The receptor-mediated uptake of proteins by APCs has been shown to produce a quantitative increase in the antigenicity of proteins (Tan *et al.*, 1997) and peptides (Agnes *et al.*, 1998) by several orders of magnitude. However, it is unclear from these studies in mice whether there are any alterations in the quality of the immune response. In similar systems, it has been demonstrated that maleylated proteins are preferentially endocytosed by scavenger receptors on macrophages (Abraham *et al.*, 1997; Abraham *et al.*, 1995; Singh *et al.*, 1998). In these instances, it has been shown that T-cell cytokine profiles become biased towards a Th1 response (Singh *et al.*, 1998). This is consistent with increased epitope density favoring a Th1 response, but is at odds with the view that glycosylation contributes to polarization towards a Th2 response.

Together, these findings support the hypothesis that APCs process glycoproteins particularly effectively, and thereby mediate an enhanced immune response. Whether glycosylation can bias the response raised against a protein towards a dominant Th2 phenotype *in vivo* is as yet unknown. It is nevertheless interesting to note that type 2 cytokines have been found to increase the expression and activity of mannose receptors on APCs (Longoni *et al.*, 1998; Stein *et al.*, 1992). This suggests that a feedback loop may exist that enhances the uptake of glycosylated proteins.

Biologic Activity of Allergens

It has been known for some time that proteins with enzymatic activity have a propensity for inducing allergic reactions (Baur *et al.*, 1982; Dijkman *et al.*, 1973; Flindt, 1969; Pepys *et al.*, 1969; Pham and Mire, 1978; Quirce *et al.*, 1992; Weill *et al.*, 1971). From an evolutionary perspective, it is reasonable that untoward extracellular enzymatic activity might provoke an immune response, as it likely reflects the presence of infec-

tious agents against which such a response is appropriate. Where the presence of enzymatic activity is likely to reflect the activity of organisms against which a type 2 immune response is appropriate, it is similarly not surprising that an immediate-type hypersensitivity reaction may result. The question then arises as to how enzymes may trigger such allergic reactions.

Allergens possess a wide range of biologic activities. For some, mechanisms have been identified whereby the activity could contribute directly to the efficacy of the allergen. Perhaps the best studied activity is that of the house dust mite allergen, Der p 1, which is known to have proteolytic activity. This activity has been shown to augment the permeability of the bronchial epithelium, and so may contribute to the uptake of the allergen and the production of inflammatory cytokines (Herbert *et al.*, 1995). Der p 1 is also able to cleave CD23 (Fc ϵ RII, the low-affinity IgE receptor) and CD25 (IL-2 receptor α -chain) expressed on the surface of some leukocytes (Hewitt *et al.*, 1995; Schulz *et al.*, 1995), leading to dysregulation of the immune response and enhanced production of IgE (Shakib *et al.*, 1998). Other proteases may use at least some of these strategies to similar effect.

The direct stimulation of mast cells in an IgE-independent manner by protein allergens has been reported in a number of different systems (Dudler *et al.*, 1995; Emadi-Khiav and Pearce, 1996; Helm, 1994; Machado *et al.*, 1996), which by implication could enhance an allergic response *in vivo*. Nevertheless, the allergenicity of one of these allergens, phospholipase A 2 (the major allergen in bee venom), has been found by one group to be unaffected by mutations that render it enzymatically inactive (Wymann *et al.*, 1998).

Clearly, although some proteins possess biologic activities that may enhance their allergenicity, there are many instances where such activity is apparently irrelevant. Of course, it may be that some allergens just happen to be enzymes and their biologic activity is irrelevant. It is tempting to speculate, however, whether the constraints placed upon a protein that permit it to be an enzyme may also predispose it towards being an allergen. One possibility is that many enzymes are relatively stable in hostile environments in order to permit them to carry out their functions effectively; the correlation between stability in SGF and allergenicity has been discussed earlier in this review. Another possibility arises from the *modus operandi* of enzymes; typically, they bind substrates in specialized, often hydrophobic, pockets that are accessible from the surface of the protein. These pockets may have unusual antigenic potential, perhaps contributing towards allergenicity. The access of substrate to these pockets normally depends upon the flexing of the enzyme; such flexibility may also contribute towards allergenicity, perhaps by facilitating the binding of B-cell receptors or IgE. It will be interesting to determine whether mutations that have little effect on the active sites of an enzyme are less likely to reduce its allergenicity than those that severely compromise the active sites.

Conclusions

The occurrence of an allergic reaction to a protein depends upon a complex interplay between the immune system and the protein. There appears to be a continuum of sensitizing activity among proteins, indicating that allergenicity *per se* may have no single, common, structural cause. Most likely, many different features can make some contribution toward overall allergenicity. Strong allergens, rather than possessing one or two strongly allergenic features, may possess many proallergenic features that singly have little effect, but which in concert can produce a powerful overall effect. The challenge is to chart and quantify the contribution that each structural feature contributes to protein allergenicity.

Many structural features of proteins could potentially contribute towards their allergenicity. These may be classified as belonging to one of two groups: those that affect the identity of the epitopes within the protein, and those that affect the availability of these epitopes to the immune system. Clearly, the identity of T epitopes defined by the primary amino acid sequence can affect the quality of the immune response to a protein, although whether such effects translate to measurable alterations in allergenicity of proteins remains to be tested. There is clearer evidence that other structural features of proteins, such as stability in SGF, the presence of glycosyl groups, and enzymatic activity, can influence allergenicity, although in many instances the underlying mechanisms remain unclear.

The current understanding of the characteristics that confer upon proteins the ability to induce allergic responses in susceptible individuals is still rather rudimentary. However, some important variables are being identified and explored, which in the future should contribute to a better appreciation of the important toxicologic challenges that protein allergenicity poses—hazard identification, potency evaluation, and risk assessment.

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