CHAPTER 1

Overview of Mucosal Immunity and Development of Oral Tolerance

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KEY CONCEPTS

- The GI mucosa is the major immunologic site of contact between the body and the external world.
- The manner in which immune cells encounter antigen determines the subsequent immunologic response.
- Oral tolerance is a complicated process, probably proceeding by several overlapping mechanisms.
- Many factors, including developmental stage, microbial exposures, diet and genetics, influence the balance between allergy and tolerance.

Introduction

The mucosa is the principal site for the immune system's interaction with the outside environment. Unlike the skin, which is characterized by many layers of stratified epithelium, the intestinal mucosa is lined with a single layer of columnar epithelium. Almost two tons of food travel past this thin barrier each year. More than one trillion bacteria representing about 500 distinct species live in contact with it. The vast majority of these bacteria are nonpathogenic commensals, but pathogens lurk in this diverse antigenic stew, and even the commensal bacteria have the potential to cause harm if not kept in check. The mucosal immune system performs the essential job of policing this boundary and distinguishing friend from foe.

Not only must the mucosal immune system determine the local response to an antigen, but, as the primary site of antigenic contact for the body, it also plays a central role in directing the systemic response to antigens. Oral tolerance – the modulation of the immune response to orally administered antigens – is a fundamental task of the mucosal immune system. In general, as befits the ratio of benign to pathogenic antigens it encounters, the default response of the mucosal immune system is tolerance. The tendency to tolerize to fed antigen can even be used to overcome already developed systemic sensitization, something known and exploited long before the specific cells comprising the immune system were identified. Yet, despite the general bias toward tolerance, the mucosal immune system is capable of producing protective responses to pathogens. This response is controlled by recognition of inherent characteristics of the antigen, or contextual cues such as tissue damage. In general, the immune system is remarkably skilled at responding properly to the antigens it encounters. Failures, albeit uncommon, can be very serious. Food allergy is a prime example of the failure of oral tolerance.

How the mucosal immune system determines when to sound the alarm and when to remain silent is the focus of this chapter. In it, we examine

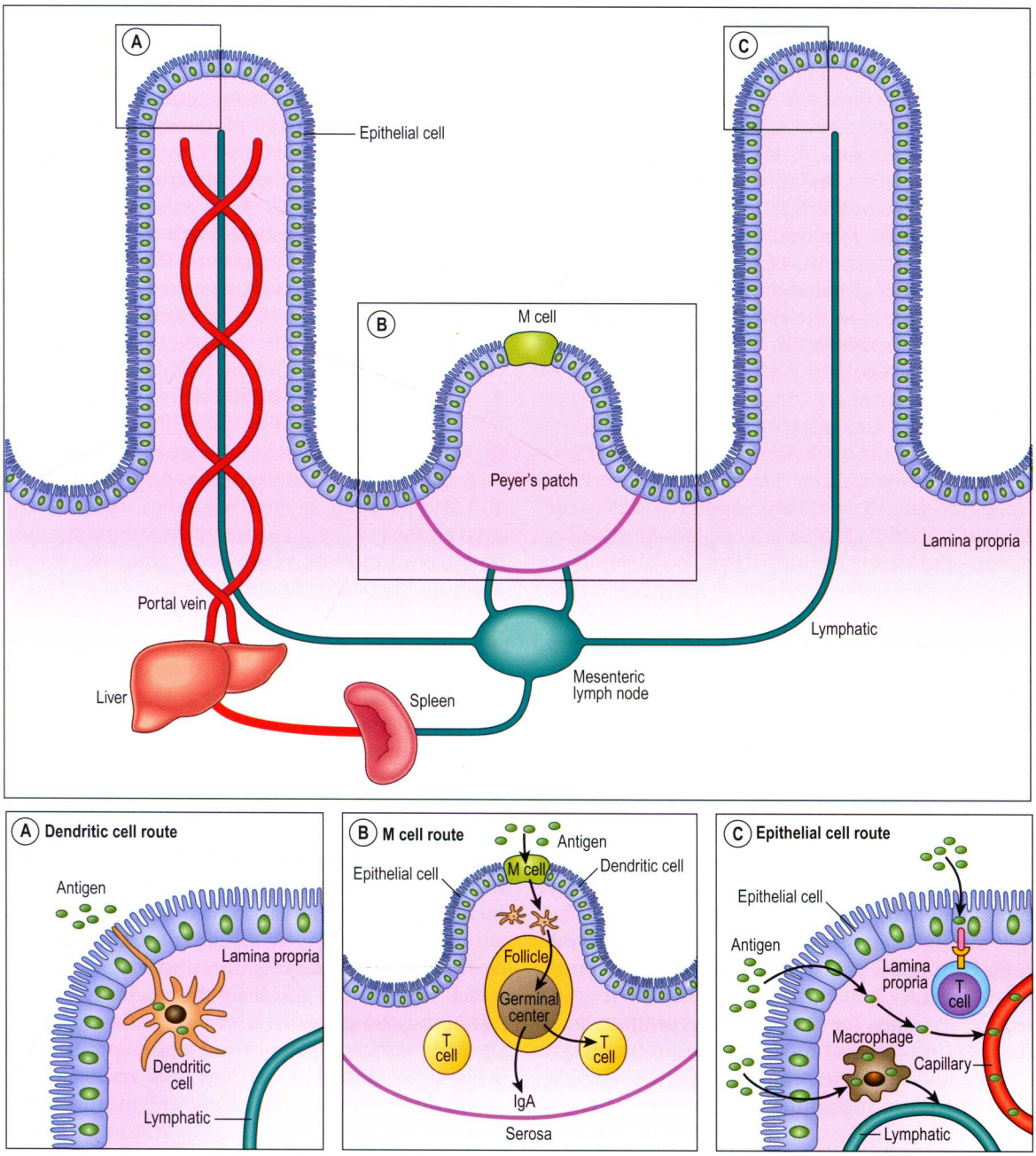

Figure 1.1 Antigen sampling in the gut. (A) Dendritic cells sample antigen directly by extending processes into the lumen. (B) Antigen taken up by M cells travels to the underlying Peyer's patches. (C) Antigen can cross the epithelium for transport to antigen-presenting cells, T cells, or into the lymphatic circulation. Reproduced with permission from: Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol 2005; 115: 3–12.

crypt.[2,4] Cytokines associated with both autoimmune and allergic disease disrupt barrier function and increase permeability.[5] Children with food allergy have been shown to have increased intestinal permeability, both at a time when they are regularly consuming the relevant allergen and after a long period of avoidance.[6,7] Other evidence for the importance of barrier function in allergy is the high rate of new sensitization in people taking the anti-rejection medicine tacrolimus, which causes mucosal barrier dysfunction. Although tacrolimus has other effects on the immune system, the high rate of new food allergies after solid organ transplantation is thought to be due its effects on mucosal integrity.[5]

In addition to the paracellular route, several alternative transport systems actively carry proteins, electrolytes, fatty acids and sugars across cells. Specialized modified epithelial cells called M (or microfold) cells act as non-professional antigen-presenting cells. These cells stud the follicle-associated epithelium overlying specialized collections of immune cells called Peyer's patches. They express receptors that recognize microbial patterns and aid in the endocytosis and transfer of antigen to the basal surface of the epithelium. This is especially important for bacteria, but may also be relevant for food allergens.[4]

Other non-specialized columnar epithelial cells form vesicle-like structures that allow transport of dietary proteins across cells. The formation of these vesicle-like structures seems to be dependent on MHC class II binding, but transocytosis can also occur via binding of antigen to IgA, IgE, and IgG. Transport via IgE may be especially important in the acute allergic response and in the amplification of allergy.[4] In contrast, secretory IgA, which accounts for the majority of the immunoglobulin produced by the body, complexes with antigen and facilitates transport across the epithelium to antigen-presenting cells, with a tolerogenic outcome.

A final method of antigen transport involves direct sampling of the luminal contents by extensions of antigen-presenting cells. Dendritic cells found in the lamina propria form their own tight junctions with intestinal epithelial cells and can project directly into the intestinal lumen. These projections increase when invasive bacteria are present, and sampling via this route seems to be especially important for the transport of commensal and invasive bacteria.[4]

Initial contact with the mucosal immune system

Once the antigen has been captured by dendritic cells, either by direct sampling or after processing through epithelial cells, the fate of the immune response depends on the interaction between dendritic cells and naive CD4+ T cells. Of the professional antigen cells associated with the gut, dendritic cells are the most important. They are found throughout the mucosal-associated lymph tissue and comprise a large class of phenotypically and functionally diverse cells. Subspecialization of these cells is thought to depend on their derivation (some develop from lymphoid precursors and some from myeloid precursors), their maturity, and environmental cues. This interaction can occur in specialized aggregations of antigen-presenting cells, T cells and B cells, such as Peyer's patches, in the loose aggregations of lymphocytes in the lamina propria, or, most importantly for food antigens, in the draining mesenteric lymph nodes.

Although there is communication between the mucosal and systemic immune systems, contact that is essential for both protective immune responses and oral tolerance, there is significant compartmentalization of responses at the mucosal level. The mesenteric lymph nodes act as a 'firewall', keeping the systemic immune system ignorant of much of the local immune response. In animals whose mesenteric lymph nodes have been removed, massive splenomegaly and lymphadenopathy develop in response to typical exposure to commensal organisms. In fact, much of the interaction with commensal organisms never even reaches the level of the mesenteric lymph nodes. IgA+ B cells, which collectively produce the majority of the immunoglobulin in the body, are activated at the level of the Peyer's patches and lamina propria and act locally. Induction of this IgA response can proceed normally in mice deficient in mesenteric lymph nodes. Although the response to commensals happens largely at the level of the Peyer's patches and lamina propria, for food antigens it seems that the mesenteric lymph nodes are key for the active response that constitutes oral tolerance. Mice without Peyer's patches develop oral tolerance normally, but those without mesenteric lymph nodes cannot. For food antigens, it seems that the typical path is for dendritic cells in the lamina propria to traffic to the mesenteric lymph nodes for presentation to CD4+ cells.[7,8]

Different experimental models have shown somewhat different kinetics of traffic to mesenteric lymph nodes after oral antigen. However, within days after exposure, dendritic cells carry orally fed antigen to the mesenteric lymph nodes and cause T-cell proliferation. T cells stimulated in this way then travel back to the mucosa and to the systemic lymph nodes.[9]

Once captured and processed, antigen presented by dendritic cells can cause several distinct immune responses. It is this interaction that determines whether allergy or oral tolerance develops.

What is oral tolerance?

Before we can begin to discuss what factors influence the development of oral tolerance, we must discuss what is meant by oral tolerance. There is disagreement at a fundamental level about how oral tolerance to foods develops. Not only are the specific mechanisms of oral tolerance imperfectly understood, but also the overall paradigm. Here we explore different theories about the development of oral tolerance.

Immune deviation

Starting in the 1980s, with work from Coffman and Mosmann, researchers began to describe distinct subsets of CD4+ T cells that were characterized by distinctive cytokine milieus and resulting disease or protective states.[10] A central paradigm in immunology for the past two decades has been this division of effector CD4+ T cells into Th1 and Th2 cells, both responsible for different mechanisms of clearing infection and both causing different pathological states when overactive. The cytokines that Th1 cells secrete (such as IFN-γ) activate macrophages and facilitate clearance of intracellular pathogens. In contrast, Th2 cells produce cytokines that promote class switching and affinity maturation of B cells, and signal mast cells and eosinophils to activate and proliferate. Th2 responses are important for clearance of extracellular parasites.

Allergy is dominated by the Th2 response and is characterized by IgE production, eosinophilia, mast cell activation, and, in some cases, tissue fibrosis. For many years it has been posited that the central defect in allergy is an imbalance between Th1 and Th2 responses. This model, although an oversimplification, has proved helpful in identifying factors that promote allergy. In the original model naive T-helper cells were stimulated by dendritic cells to develop either as Th1 or Th2 cells. Cytokines necessary and sufficient for Th1 polarization include IL-12 and INF-γ, but the mechanisms of Th2 differentiation have remained elusive. Two cytokines, IL-4 and IL-13, play a role, but are not essential for the development of high numbers of Th2 cells in the mouse model. Until recently, a leading hypothesis was that Th2 differentiation is the default response that occurs in the absence of Th1-directing signals. The theory of Th2 as a default has appeal because it harmonizes nicely with the so called 'hygiene hypothesis', in which inadequate infectious stimuli create the conditions for allergy. If Th2 deviation were the default, allergic responses would naturally develop in the absence of Th1 driving infectious stimuli. Recent work, however, suggests that Th2 differentiation requires other signals, including OX40L from dendritic cells, but that the signals essential for Th1 differentiation are stronger and predominate if present.[11]

Despite the compelling qualities of this theory, it is now clear that the reality is much more complicated. Although allergy is characterized by a Th2 response, an increasing body of evidence calls into question whether it is simply the balance between Th1 and Th2 responses that lies at the crux of the problem of allergy. Epidemiologic studies do not consistently show a reciprocal relationship between incidence of Th1 imbalance (i.e. autoimmunity) and Th2 imbalance.[12] Adoptive transfer of Th1 cells in mice cannot control Th2-induced lung inflammation.[13] A recent study showed that allergic subjects had low-level Th1-type cytokine responses to allergenic stimulation that matched the non-allergenic responses but were simply overwhelmed by the massive Th2 cytokine response.[14] Most importantly, other types of CD4 cells important in the control of both allergy and autoimmunity have been identified.

Regulatory T cells

The existence of T cells with suppressive capacity was first recognized in the 1980s. Initially, centrally derived T-regulatory cells were identified. These cells are important in regulating autoimmunity and are generated in the thymus, in a process of T-cell selection that has been compared to Goldilocks' sampling of the bears' oatmeal. T cells with too strong an attraction to self antigens are deleted, as

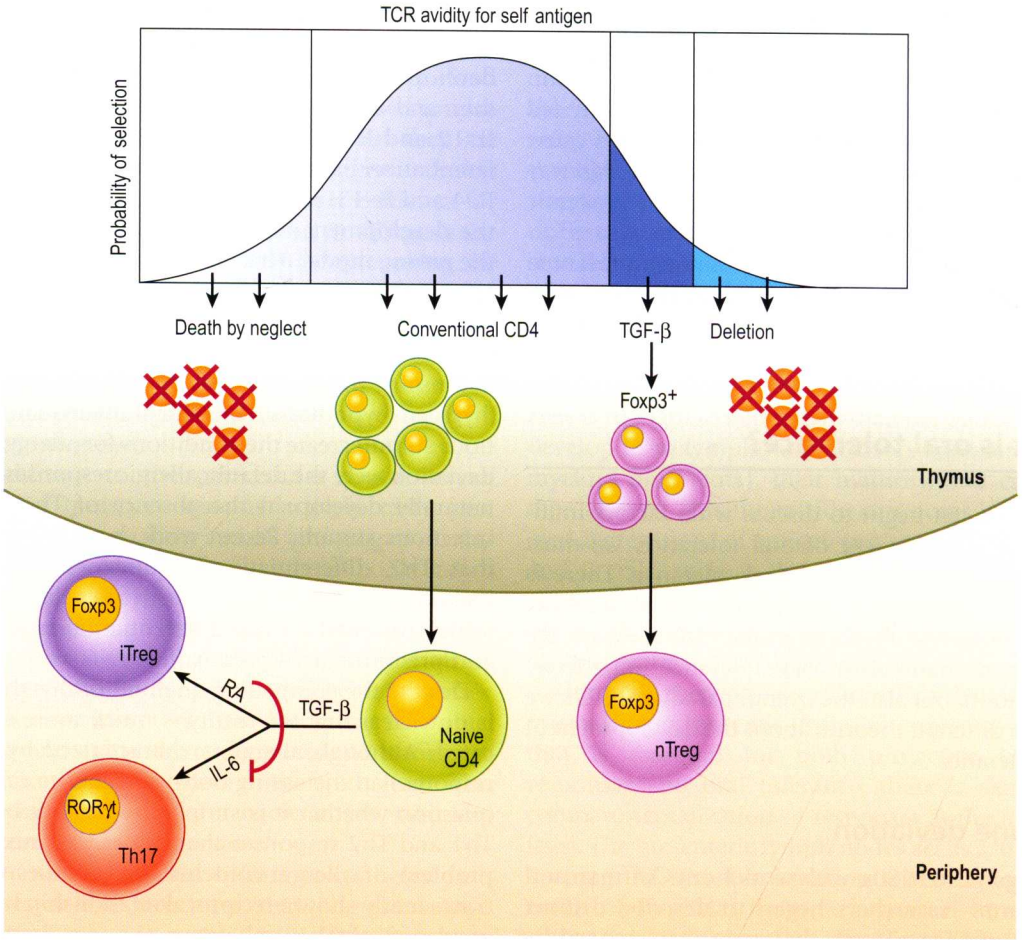

Figure 1.2 The development of regulatory T cells. In the thymus, avidity of the T-cell receptor for self antigen determines the fate of the T cell. In the periphery, naive Foxp3– CD4+ T cells can develop into FoxP3+ T-regulatory cells or Th17 cells, depending on the cytokine milieu. Reproduced with permission from: Mucida D, Park Y, Cheroutre H. From the diet to the nucleus: vitamin A and TGF-beta join efforts at the mucosal interface of the intestine. Semin Immunol 2009; 21: 14–21.

are those that do not bind well at all, and thus will not be effective antigen presenters. The majority of the remaining cells bind 'just right' at a moderate level and are destined to become effector T cells, but a subset that binds to self antigens more strongly persists and becomes suppressive T cells (Fig. 1.2).[15] A transcription factor, FOXP3, is essential for the suppressive nature of these cells and has served to identify them. The importance of these cells in autoimmune disease has been amply demonstrated, both in animal models – autoimmune disease can be induced by depletion of these cells – and in natural human diseases. Children with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome have mutations in the FOXP3 gene leading to absent or abnormal levels of regulatory T cells. These children have early and severe autoimmune gastrointestinal and endocrine disease. Bone-marrow transplant that replaces the T-regulatory cells successfully reverses the disease.

Children with IPEX also have food allergy and eczema, demonstrating a failure of tolerance to antigens that are not present in the thymus. More recently, the importance of peripherally generated T-regulatory cells has become clear. As with the centrally generated T-regulatory cells, FoxP3 marks these cells (called iTregs), although other related

subsets of suppressor T cells generated in the periphery do not express Fox P3. T-regulatory cells are preferentially induced in the mesenteric lymph nodes, where the cytokine TGF-β is a key mediator of T-cell differentiation. In the past decade, it has been determined that T-regulatory cells and a newly described T-cell subset, Th17 cells, develop reciprocally under the influence of TGF-β. A cytokine, IL-6, drives differentiation to Th17 cells, whereas a metabolite of vitamin A, retinoic acid, was recently discovered to inhibit Th17 differentiation and promote T-regulatory development in the presence of TGF-β.[16] Vitamin A, which is not produced by the human body, is converted to its active form, retinoic acid, by epithelial cells and dendritic cells. The fact that generation of suppressor cells is dependent on an orally derived factor that is converted to an active form by the intestinal epithelium may help explain how the gut is maintained as a tolerogenic site.[17]

Peripherally generated T-regulatory cells have a multitude of effects on other immune cells. Through the action of secreted cytokines, such as IL-10 and TGF-β, they act on B cells, reducing IgE production and inducing the blocking antibody IgG4; on Th1 and Th2 cells, suppressing their inflammatory activities; and on dendritic cells, inducing them to produce IL-10 and further stimulate the development of regulatory T cells. In addition, they have direct interaction with mast cells through cell surface ligands (Fig. 1.3). In sum, they control both Th1- and Th2-mediated inflammatory responses.[18]

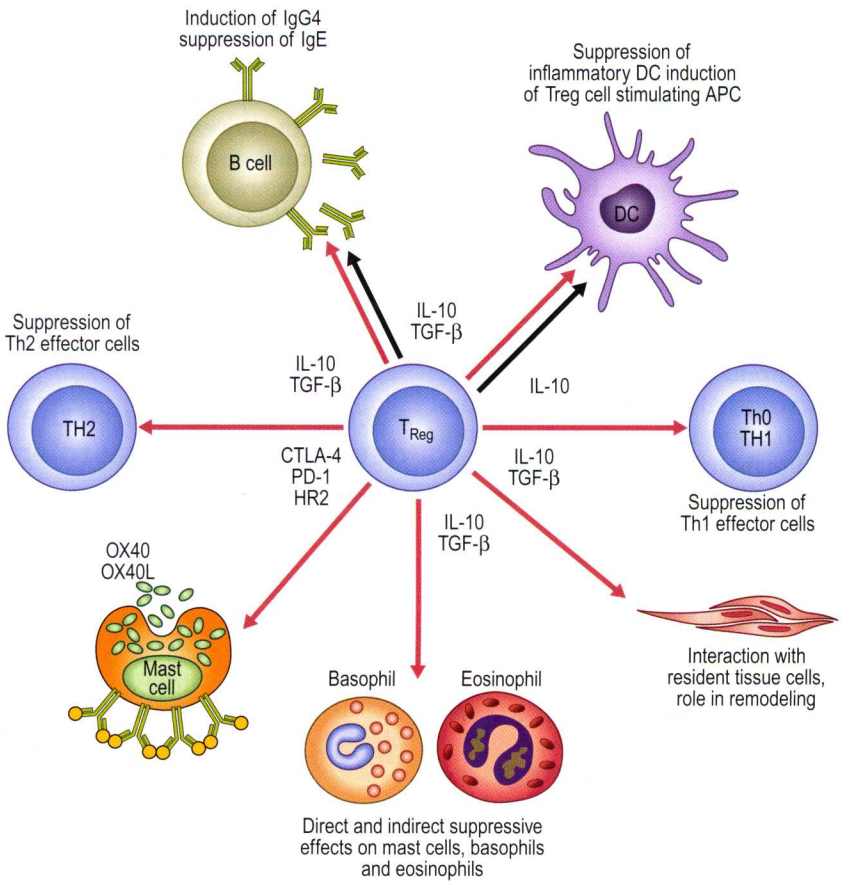

Figure 1.3 T-regulatory cells have direct and indirect effects on many different types of effector cells. Suppressive cytokines include interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). Another mechanism of suppression is by cell–cell contact via OX40-OX40ligand (red arrows: suppression; black arrows: induction). Reproduced with permission from: Akdis M. Immune tolerance in allergy. Curr Opin Immunol 2009; 21: 700–7.

of risk and the optimal strategy to prevent allergy in infants are among the most contentious areas in the field of allergy.

Part of the difficulty of resolving these controversies lies in the inadequacy of the animal models. Both human and rodent neonates have increased intestinal permeability compared to their adult counterparts. However, in humans, the transition from the highly permeable fetal gut to a more mature gut barrier occurs in the first few days of life, compared to more than a month in rats.[25]

One well-studied area is the difference in gastric pH and pancreatic enzyme output between infants and adults. With their immature barriers to regurgitation of caustic gastric contents, infants secrete much less acid into the stomach and have decreased pancreatic enzyme output, and do not reach adult levels of pH for the first few years of life.[25] As discussed above, acidic and enzymatic digestion is a first-line defense preventing some potentially sensitizing proteins from reaching relevant immune cells. Combined with somewhat increased intestinal permeability, this increases the chances of intact allergen crossing the epithelial border.

Once across the epithelial border, the immune system that the antigen encounters is very different in neonates than in adults. Both cellular and humoral branches of the immune system are immature. Total numbers of dendritic cells are lower, as is their ability to respond to co-stimulatory factors that typically elicit a Th1-type response. Further, CD4+ T cells are themselves highly skewed in a Th2 direction in the neonate, and have poor production of IL-12, a cytokine involved in Th1 responses. The inability to mount Th1 responses but ability to mount Th2 responses leads to an environment where potential autoimmunity or reactivity to maternal antigens is dampened, responses to microbial insults are deficient, and allergic responses are relatively favored.[26]

The fetal and neonatal immune system is also characterized by varying levels of T-regulatory cell function. At the time of birth, T-regulatory cells are found less frequently in cord blood than in adult blood, and those found have less efficient suppressive function after stimulation.[28] However, there is some evidence that, at least in mice, neonatal T cells have a propensity to develop into T-regulatory cells.[27] Given the uniquely stressful experience of birth, one could question whether what is found in cord blood is a valid reflection of the intrinsic qualities of the neonate. Regardless, the T-regulatory cell compartment is one area where neonatal and adult responses vary considerably, with important implications for the development of allergy.

The humoral immune system is also immature in the infant. Immaturity of the humoral immune system is at least partially compensated for by unique features of breast milk. Breast milk contains large amounts of secretory IgA and some IgG. Maternally supplied IgA substitutes for the infant's relative lack, complexing with dietary proteins and promoting non-inflammatory responses.[25] IgG found in breast milk plays a similar role, with added nuances. Neonates express a receptor for IgG in their intestinal epithelium (the FcRn receptor). This allows for active transport of IgG from breast milk into the neonatal circulation. In addition to absorbing maternal antibody to be used in fighting infections, the FcRn receptor can also transport intact antigen complexed with IgG directly from the lumen to lamina propria dendritic cells, contributing to oral tolerance. In mice, antigen complexed to IgG in breast milk has been shown to induce antigen-specific T-regulatory cells in a manner independent of the other ingredients in breast milk. Interestingly, this was enhanced in mothers who were sensitized to the allergen.[29]

Other components of breast milk are important in oral tolerance. Pro-forms of the tolerogenic cytokine TGF-β are abundant in breast milk. They are thought to be physiologically active after exposure to the acidic gastric environment, and epidemiologic work in humans suggests that higher levels are associated with protection from atopic disease.[30,31]

Despite these pro-tolerogenic features, the presence of allergen in breast milk does not always lead to oral tolerance. Allergens are found both free and complexed to antibody in breast milk, and infants can become sensitized to proteins encountered in breast milk and react to them. Complicating the picture further, maternally ingested or inhaled allergens have also been found in the placenta, although whether this allergen is transferred to the fetal circulation remains unclear. Studies in mice have shown variation in the results of prenatal exposure by the dose of antigen. Mice whose mothers had low doses of prenatal exposure to a model allergen developed tolerance to that allergen. With higher doses there was transient inhibition of IgE production upon challenge, but after the immediate neonatal period the mice had increased susceptibility to the development of allergy to that allergen.[32]

Whether sensitization or oral tolerance to these antigens occurs probably depends on a complex interaction between the non-allergen components of breast milk, infant factors, and the dose and timing of the allergen.

Route of exposure

Some have suggested that the primary route of sensitization leading to food allergy is via the skin. In this model, oral exposure is almost always tolerogenic. Allergy happens when the skin encounters potentially allergenic foods prior to oral contact. Eczema, which creates breaks in the skin and an inflammatory backdrop, predisposes to allergic sensitization. Evidence supporting this model includes the fact that mice can be sensitized via low-dose skin exposure, some epidemiologic evidence tying peanut oil-containing lotions to peanut allergy, and the differences in immune responses induced by antigen-presenting cells in the skin and in the gut. However, this theory has not been conclusively proven.[33]

Microbial influences

The most compelling theory for the wide variation in incidence in allergic disease remains the so called 'hygiene hypothesis'. In general terms, this theory posits that the decreased burden of infection, especially childhood infections, characteristic of the western lifestyle does not adequately stimulate the developing immune system into a non-allergic phenotype. The beauty – and the limitation – of this theory is that it is sufficiently broad to encompass a wide range of theoretical mechanisms by which infection might prevent allergy, including Th1 skewing and induction of T-regulatory cells, and that it does not specify what infections are actually essential.

Epidemiologic evidence supporting the hypothesis includes the fact that allergy is more common in developed than in developing countries, in city than in farming communities, in children who do not attend daycare, and in older siblings than in younger siblings, especially younger siblings in large families. A thorough analysis of farming communities in Europe identified unpasteurized milk and the presence of multiple species of farm animals living under the same roof as key protective factors of the rural life. In other populations, markers for parasitic infections, such as Schistosoma, are associated with reduced rates of allergy. In addition, differences in the microbial content of drinking water have been linked to the disparate rates of atopic disease found in genetically similar populations of people living on different sides of the Finnish/Russian border. Similar epidemiologic studies also associate infection with protection from autoimmune disease.[34]

Evidence tying actual differences in gut flora to allergy has been mixed, with some finding that allergic children have different colonization patterns, and others failing to replicate the result. Birth by Caesarean section, which does not expose the infant to the normal maternal vaginal and fecal flora, has been associated with alterations in the infant's fecal flora. In one study,[35] Caesarean delivery was associated with an increased risk of wheezing, although this was not replicated in another study. Methodological problems with how gut flora were analyzed may be a part of the confusion, as the relevant bacteria may be hard to culture.

In rodent models, intestinal colonization is essential for normal development of the immune system and for the ability to induce oral tolerance. Recent work has identified certain bacterial components as being essential for the development of the normal gut immune system.[36] Specific mechanisms for prevention of allergy by infection are still being worked out. In humans, the mechanisms have been most carefully explored in prospective studies of children growing up on European farms. In these studies, several mechanisms of protection from allergy were identified, including upregulation of Toll-like receptors (TLRS), increased T-regulatory cell function and alterations in prenatal serum cytokine levels.[37–39] Prenatal farm exposure has been identified as particularly protective for the development of allergy. Whether the prenatal exposure is mediated by colonization of the infant, epigenetic changes passed from mother to child, or by so far unidentified features of the intrauterine environment, is unknown.

Nutritional factors

Nutritional factors are one way in which the prenatal environment or early life could modify the risk for allergic disease. Because diet has changed so rapidly in developed countries over the last half century, nutritional factors are candidates to explain the rapid increase in allergic disease and the geographic variation in disease. The Mediterranean diet in general during pregnancy has been associated with

could alter the microbial contents with beneficial results. Prebiotics, which contain elements that stimulate specific bacterial growth, and probiotics, which contain the bacteria themselves, have been used in many small studies for the prevention and treatment of allergic disease. In sum, the studies suggest a small beneficial effect for the prevention of atopic dermatitis, but no benefit for the treatment of established disease or for the prevention of other atopic conditions. Larger, well-designed studies are required before probiotics can be confidently recommended.[50]

Other dietary factors are promising, although they have not yet been fully evaluated. As discussed above, the single randomized controlled study of fish oil found some protection from food allergy, but this needs to be replicated. It is not yet clear whether an increase or reduction in vitamin D and folic acid would be the best intervention for prevention of food allergy. Well-designed prospective epidemiologic studies are the first necessary step to sort this out.

Conclusions

Oral tolerance is a complex, active process that occurs in the gut-associated immune system. Although the precise mechanisms have not been completely elucidated, regulatory T cells seem to be essential for its development and maintenance. Other, overlapping mechanisms, including immune deviation, anergy and deletion, also play a role. Many factors affect the balance between allergy and oral tolerance. They include genetic variations, the dose, timing and route of antigen exposure, the microbial milieu, and probably other dietary factors. This field is still young, and much remains to be done to identify the mechanisms of allergic sensitization. Because of the complexity of the system, some things will not be known until interventional studies in humans are carried out.

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CHAPTER 2

Food Antigens

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Introduction

The immune system possesses remarkable flexibility in the number of ways in which it works to protect the body from hazards, including infective microorganisms, viruses and parasites, employing both cellular agents to remove and inactivate hazards, as well as molecules, notably immunoglobulins (Igs), which form part of the humoral defense system. Igs are synthesized in a number of different forms, or isotypes, and have been classified on a structural, physicochemical and functional basis including IgA, IgG (of which there are a number of subtypes identified in humans, including IgG_1 and IgG_4), IgM and IgE. All are characterized by an antibody-binding site generated to bind specifically to 'non-self' molecules, which are generally known as antigens. These include molecules found in microbial pathogens, parasites, environmental agents such as pollen and dietary proteins. Albeit not exclusively so, antigens tend almost entirely to be proteinaceous in nature, although some carbohydrate moieties can be recognized, and the lipopolysaccharide antigens of microbes are particularly effective elicitors of humoral immune responses.

However, in the allergic condition classified as a type I hypersensitivity reaction, the antibody repertoire to selected environmental antigens is altered, the body synthesizing larger quantities of the antibody isotype normally produced in response to parasitic infections, IgE. The molecules recognized by IgE are frequently termed allergens and, if polyvalent in nature, they may be able to cross-link mast-cell-bound IgE and in so doing trigger mediator release, the inflammatory mediators then going on to trigger tissues responses which are manifested as allergic symptoms in an allergic reaction.

The sites that an antibody recognizes on its cognate antigen have been termed epitopes and can be classified into two different types. The first of these have been termed continuous or linear epitopes and are where antibody recognition is based almost entirely on the amino acid sequence, with very little effect of conformation. In general such antibodies can bind well to short linear peptides of 10–15 residues in length that correspond to the epitope sequence in the parent protein. They also often recognize both native folded and unfolded antigens well. A second type of epitope has been termed conformational and is where the secondary, tertiary and quaternary structural elements of a protein antigen bring together sometimes quite distant regions of the polypeptide chain. In general, antibody binding to such epitopes is disrupted when proteins unfold, and it can be difficult to map such epitopes using linear peptides as they do not resemble the structural epitopes brought about by the folded nature of the antigen. Structural studies have indicated that antibody binding to proteins involves a surface area of 650–900 $Å^2$, contacts outside the immediate epitope

area being important in binding although they may not determine antibody specificity. Such definitions are in some ways arbitrary, and it may be in some instances that several linear epitopes could come together to form a conformational epitope.

Allergens have been defined by the International Union of Immunological Societies as being molecules that must induce IgE-mediated (atopic) allergy in humans with a prevalence of IgE reactivity above 5%. Although it does not carry any connotation of allergenic potency, an allergen is termed as being major if it is recognized by IgE from at least 50% of a cohort of allergic individuals, otherwise it is known as minor. Allergens are given a designation based on the Latin name of the species from which they originate and composed of the first three letters of the genus, followed by the first letter of the species and finishing with an Arabic number. Thus, an allergen from *Mallus domesticus* (apple) is prefaced Mal d followed by a number, which is largely determined by the order in which allergens are identified. The numbers are common to all homologous allergens (also known as isoallergens) in a given species, which are defined on the basis of having a similar molecular mass, an identical biological function, if known, e.g. enzymatic action, and >67% identity of amino acid sequences. For those species where the first three letters of a genus and the first letter of a species are identical, the second letter of the species is also used.

Many proteins are post-translationally modified with glycans and such structures can bind IgE, glycan-reactive IgE being found in between 16% and 55% of food-allergic patients. These are best characterized for the asparagine-linked carbohydrate moieties (*N*-glycans), with α(1–3) fucose and β(1–2) xylose representing the major cross-reactive carbohydrate determinants (CCDs), which are found in many plant food and pollen allergens but are distinct to mammalian *N*-glycans. However, there is debate about whether IgE to CCDs has biological significance, and whether it can result in clinically significant allergic symptoms. This is probably because such glycans tend not to be polyvalent, and consequently are unable to trigger cross-linking of IgE receptors, the IgE binding may be of low IgE affinity, and the presence of blocking antibodies may downregulate the allergic response. *O*-linked glycans are also found in plant proteins, albeit less frequently than *N*-glycans. There is evidence that single β-arabinosyl residues linked to hydroxyproline residues are important in determining the IgE-binding activity of an allergen from mugwort pollen known as Art v 1, although *O*-linked glycans have yet to be described in food allergens.

In the process of describing the active agents involved in food allergies a large number of allergens have now been identified with the greatest diversity existing for plant food allergens, perhaps reflecting the diversity of plant-derived foods that humans consume. They include nuts, seeds, grains, and a variety of fresh fruits and vegetables. Although it appears that individuals can be allergic to any of a vast number of foods, it appears that the majority of allergies are triggered by a more restricted selection, and that the allergens triggering those reactions belong to a restricted number and type of protein. This observation has led to certain restricted numbers of foods being termed the 'Big 8' which includes milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybean. Other important allergenic foods or food groups have emerged, some of which, along with the 'Big 8' must be labeled on manufactured foods in certain countries and regions of the world to allow allergic consumers to avoid them. These include molluscan shellfish, mustard, celery (root celery or celeriac) and lupin. This review will focus on the structural attributes and common properties of allergens and then describe in more detail the allergens found in more commonly important allergenic foods.

Common properties and structural attributes of food allergens

The last 10–15 years have seen an explosion in the number of allergenic proteins described from a vast array of foods, which has allowed the application of various bioinformatic tools to classify them according to their structure and function into protein families. Some years ago this was undertaken for both plant and animal food allergens, together with pollen allergens. This analysis has demonstrated that the majority of allergens in each of these groups fell into around three to 12 families, the remaining allergens belonging to around 14–23 families comprising one to three allergens in each. Thus, around 65% of plant food allergens belonged to just four protein families, known as the prolamin, cupin, Bet v 1 and profilin superfamilies, whereas animal-derived food allergens fall into just three main families, namely the tropomyosins, EF-hand

and caseins. A summary of the major and several of the minor allergen families is given below.

Animal food allergen families

Tropomyosins (Fig. 2.1)

Tropomyosins are contractile proteins which, together with the other proteins actin and myosin, function to regulate contraction in both muscle and non-muscle cells and are ubiquitous in animal cells. They comprise a repetitive sequence of heptapeptide repeats that spontaneously form two strands of α-helix which then assemble into two-stranded coiled coils. These monomers then assemble into head-to-tail polymers along the length of an actin filament. These are the major allergens of two invertebrate groups, Crustacea and Mollusca, which include the food group commonly known as shellfish. They have been identified as both food and inhalant allergens, being characterized as allergens in dust mite and cockroach, and consequently have been termed invertebrate pan-allergens. IgE-epitope mapping has shown that sequences unique to invertebrate tropomyosins, located in the C-terminal region of the protein, play an important role in their allergenic potential. Their lack of homology between vertebrates and invertebrates means there is no cross-reactivity between IgE from shellfish-allergic individuals and animal muscle tropomyosins.

Parvalbumins (Fig. 2.2)

Parvalbumins represent the second-largest animal food allergen family and are abundant in the white muscle of many fish species, where they have a role regulating free intracellular calcium levels, which are important for muscle fiber relaxation. They are ubiquitous in animals and have been classified into two different types, α and β, which possess distinct evolutionary lineages but are structurally very similar. In general it is the β-parvalbumins that are allergenic. Structurally they are characterized by a calcium-binding motif found in many proteins, known as an EF-hand, which comprises a 12 amino

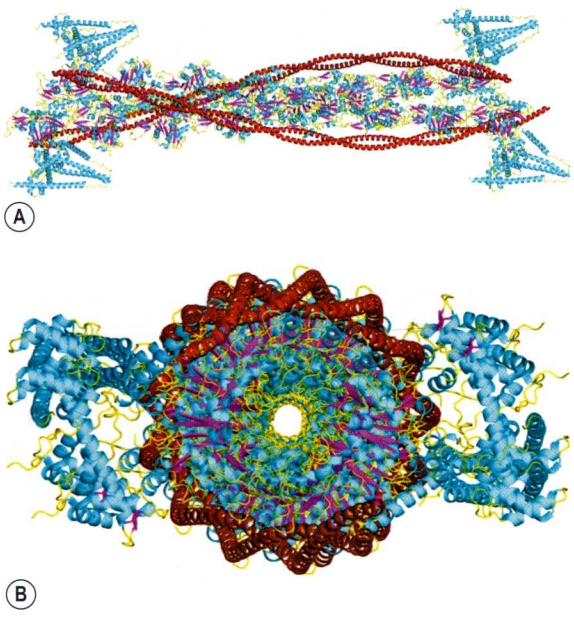

Figure 2.1 Three-dimensional structure of tropomyosin in insect flight muscle (PDB code 2W4U) and example of a tropomyosin from an invertebrate which is typical of the allergenic tropomyosins found in crustaceans and molluscs. (a) A view along tropomyosin chains; (b) a cross-sectional view. Tropomyosin is shown in red. Other proteins are troponin and actin. α-Helices and loops are shown in cyan and yellow, respectively.

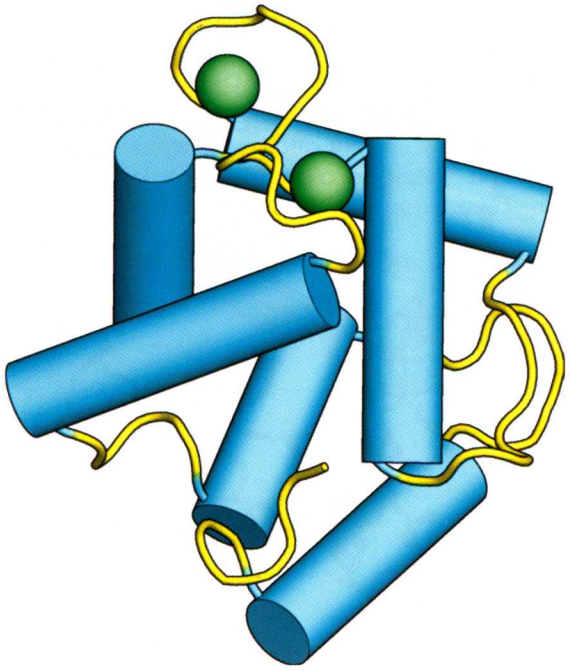

Figure 2.2 Three-dimensional structure of calcium-liganded carp parvalbumin (PDB code 4CPV, Cyp c 1). Parvalbumin has two calcium-binding sites which have the same structural motif formed by an α-helix linked to a second α-helix by a 12-residue loop around the calcium cation. Calcium cations are shown as green spheres. α-Helices are shown in cyan cylinders and loops are shown in yellow.

A second type are the Kazal inhibitors, which also inhibit serine proteases and can contain between 1 and 7 Kazal-type inhibitor repeats.

Plant food allergen families

Prolamins (Fig. 2.4)

The prolamin superfamily was initially identified on the basis of a conserved pattern of cysteine residues found in the sulfur-rich seed storage prolamins, the α-amylase/trypsin inhibitors of monocotyledonous cereal seeds, and the 2S storage albumins. Subsequently other low molecular weight allergenic proteins have been identified as belonging to this superfamily, including soybean hydrophobic protein, non-specific lipid transfer proteins and α-globulins. The conserved cysteine skeleton comprises a core of eight cysteine residues that includes a characteristic Cys–Cys and Cys–X–Cys motif (X representing any other residue). Two additional cysteine residues are found in the alpha-amylase/trypsin inhibitors. Apart from the seed storage prolamins, which are characterized by the insertion of an extensive repetitive domain, members of this superfamily share a common three-dimensional structure. This comprises a bundle of four α-helices stabilized by disulfide

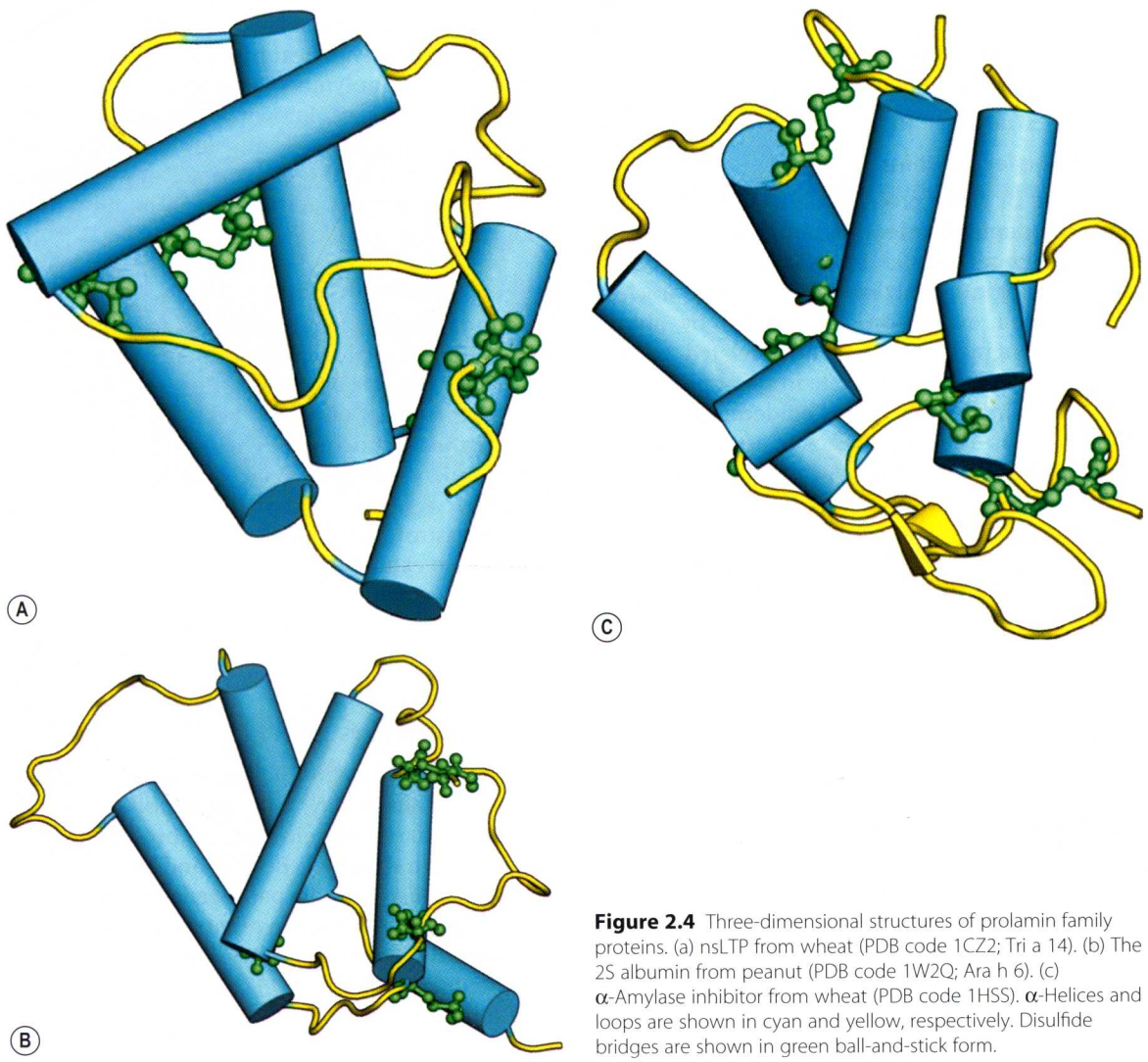

Figure 2.4 Three-dimensional structures of prolamin family proteins. (a) nsLTP from wheat (PDB code 1CZ2; Tri a 14). (b) The 2S albumin from peanut (PDB code 1W2Q; Ara h 6). (c) α-Amylase inhibitor from wheat (PDB code 1HSS). α-Helices and loops are shown in cyan and yellow, respectively. Disulfide bridges are shown in green ball-and-stick form.

bonds which are arranged in such a way as to create a lipid-binding tunnel in the nsLTPs which is collapsed in the 2S albumin structures. It is also responsible for maintaining the three-dimensional structure of many of these proteins even after heating, which is associated with their retaining their allergenic properties after cooking and may contribute to their resistance to proteolysis.

2S albumins

A major class of seed storage proteins, the 2S albumins are usually synthesized in the seed as single chains of 10–15 kDa which may be post-translationally processed to give small and large subunits which usually remain joined by disulfide bonds. The type of this processing depends on the plant species,those in sunflower being single-chain albumins and those in Brazil nut being two-chain albumins. They can act as both occupational (sensitizing through inhalation of dusts) and food allergens.

Lipid transfer proteins

The name of these proteins derives from the fact they were originally identified in plants because of their ability to transfer lipids in vitro, but their actual biological function in plants is unclear. Because their expression is regulated by abiotic stress, belonging to pathogenesis-related protein group 14, they may have a role in plant protection. They are located in the outer epidermal tissues of plants, such as the peel of peach or apple fruits, and this, together with their lipid-binding characteristics, has led to the suggestion they are involved in transporting cutin and suberin monomers to the outer tissues of plants, where they polymerize to form the outer waxy layers. They have been termed pan-allergens and are the most widely distributed type of prolamin, being found in a variety of plant organs including seeds, fruit and vegetative tissues. Thus, in addition to being identified in many different fruits and seeds, they have also been characterized as allergens in the pollen of several plant species such as olive and Parietaria judaica as well as inhalant allergens involved in occupational allergies to dusts such as wheat flour in Baker's asthma. The IgE cross-reactivity of LTPs from the *Rosaceae* fruits has been demonstrated and related to conservation of their surface structure but to date such cross-reactivity has not been demonstrated between pollen and food allergens. Certainly allergy involving peach LTP Pru p 3, has been demonstrated to be independent of pollen LTP sensitization and is associated with much higher levels of peach Pru p 3 specific IgE, implying it is the primary sensitizing agent involved in this food allergy.

Seed storage prolamins

The cysteine skeleton and α-helical structure generally characteristic of the prolamin superfamily has been disrupted in the seed storage prolamins as a consequence of the insertion of a repetitive domain rich in the amino acids proline and glutamine. This repetitive domain dominates their physicochemical properties of the seed storage prolamins and is thought to adopt a loose spiral structure formed from a dynamic ensemble of unfolded and secondary structures comprising overlapping β-turns or poly-L-proline II structures. They are the major seed storage proteins of the related cereals wheat, barley and rye, those from wheat being able to form large disulfide-linked polymers that comprise the viscoelastic protein fraction known as gluten. These proteins are characteristically insoluble in dilute salt solutions, either in the native state or after reduction of interchain disulfide bonds, being instead soluble in aqueous alcohols.

Bifunctional inhibitors

This group of allergens are restricted to cereals, individual subunits acting as inhibitors of trypsin (and sometimes other proteinases), α-amylases from insects (including pests) or both,leading to their being termed bifunctional. These proteins can have a role as allergens in occupational allergies to wheat flour, such as baker's asthma, or in food sensitizing via the gastrointestinal tract. They were initially identified in extracts made with mixtures of chloroform and water and are often called CM proteins, but are also soluble in water, dilute salt solutions or mixtures of alcohol and water.

Bet v 1 homologs (see Fig. 2.7)

A very important group of allergens are those that are homologous to the major birch pollen allergen Bet v 1. A β-barrel protein that can bind plant steroids in a central tunnel, Bet v 1 and its homologs belong to family 10 of the pathogenesis-related proteins and may have a role in plant protection, acting as a steroid carrier, although this has not been confirmed. The conservation of both primary structure (amino acid sequence) and the molecular surfaces of Bet v 1 and its homologs explains the

allergens involved in the pollen–fruit allergy syndrome. Cytosolic proteins found in all eukaryotic cells, profilins are thought to regulate actin polymerization by binding to monomeric actin and a number of other proteins. However, only profilins found in plants, where they are highly conserved, have been described as allergens. As a consequence, profilin-specific IgE cross-reacts with homologs from virtually every plant source, and sensitization to these allergens has been considered a risk factor for multiple pollen allergies and pollen-associated food allergy. However, the clinical relevance of plant food profilin-specific IgE is still under debate.

Many of the remaining minor plant food allergen families have a role in protecting plants from pests and pathogens. Two types of enzyme family have been described as plant food allergens, including the glycoside hydrolase family 19 proteins known as class I chitinases, which are involved in latex-food allergies, and the cysteine (C1) papain-like proteases. Plant class I chitinases degrade chitin, a major structural component of the exoskeleton of insects and of the cell walls of many pathogenic fungi, and hence have a role in protecting plants against pests and pathogens. They possess an N-terminal domain that is structurally homologous with hevein, a major latex allergen, which is thought to bind chitin. As a consequence of this homology, class I chitinases from fruits such as avocado, banana and chestnut have been identified as major allergens that cross-react with IgE specific to the latex allergen Hev b 6.02. The 43-residue polypeptide chain of hevein-like domains contains four disulfide bonds, to which they owe their stability, and because of their widespread occurrence in plants have been termed pan-allergens. The cysteine proteases, to which fruit allergens belong, notably in kiwi, were originally characterized by having a cysteine residue as part of their catalytic site, although some members may have lost the capacity to act as proteases, a notable example being the soybean P34 protein, in which a glycine has replaced the active site cysteine residue.

Other minor plant food allergen families include the Kunitz/bovine pancreatic trypsin inhibitors and some lectins. The Kunitz inhibitors are active against serine, thiol, aspartic and subtilisin proteases, and in plants they probably play a role in defense against pests and pathogens. They belong to a superfamily of structurally related proteins which share no sequence similarity and which includes such diverse proteins as interleukin (IL)-1 proteins, heparin-binding growth factors (HBGF) and histactophilin. The thaumatin-like proteins (TLPs) are structurally similar to the intensely sweet-tasting protein thaumatin found in the fruits of the West African rainforest shrub *Thaumatococcus daniellii*. They are also involved in plant protection, belonging to the PR-5 family of proteins.

Common properties and predicting allergens

What does the classification of allergens into protein families tell us? Great efforts have been made to use bioinformatic methods to predict what makes some proteins allergens and not others, especially to support the allergenic risk assessment process for allergens in novel foods and genetically modified organisms destined for food use. However, it is not yet possible to predict allergenic activity in proteins, and it is clear that membership of one of a limited number of protein families is not in itself sufficient to determine allergenic activity. However, proteins from the same family often share common properties conferred by the structural features of that particular family. It seems that several factors contribute to determining whether a given atopic individual will become sensitized to a given individual. These include the genetic make-up and atopic tendencies of the exposed individual and factors such as the abundance of an allergen in a food, its structure, and the biochemical and physicochemical properties of the allergen. These include a protein's 'stability', reflecting its ability to either retain or regain its original native three-dimensional structure following treatments such as cooking, and to resist attack by proteases, such as those encountered in the gastrointestinal tract. Such stability has the potential to be modified by ligands, such as lipids and metal ions. Other factors, such as interaction with membranes, the ability to aggregate, or the presence of repetitive structures, may also influence allergenic potential. It may also be that, although glycans are not so important in triggering allergic reactions in individuals once sensitized, they may play a role in effecting sensitization in the first place. However, an understanding of structural relationships and common properties does help to explain many of the cross-reactive allergies observed and the common responses of many different types of food allergy to processes such as cooking. The following sections give a summary of the current

the allergenic properties of allergens in fruits and vegetables in different ways, and it seems that different fruit tissues may respond in different fashions. Thus, for allergens such as Bet v 1 homologs, for which the IgE-binding sites are generally conformational in nature, processing procedures that denature this protein generally result in a loss of IgE reactivity, and this is particularly true of fresh fruits, although the allergenic Bet v 1 homolog from celeriac seems to retain its allergenic activity after thermal processing. The Bet v 1 homologs also tend to be labile to gastrointestinal digestion, although there are suggestions that whereas IgE epitopes may be destroyed, the short peptides resulting from gastrointestinal digestion maybe able to act as T-cell epitopes and hence may modulate immune responses, even if not involved in elicitation.

In contrast, allergens from the prolamin superfamily appear to be both resistant to thermal processing procedures and highly resistant to gastric and duodenal digestion. Notable among these are the LTPs, which are generally highly resistant to both gastric and duodenal proteases, and it seems likely that they survive digestion in a virtually intact form, a property that has been associated with their allergenic potency. They also resist thermal denaturation, often refolding on cooling, and have been found in fermented foods and beverages such as beer (where they make an important contribution to foam stability) and wines, although combinations of low pH and heating may be sufficient to denature the protein. Similarly, TLPs appear to be stable to thermal processing, being found even in highly processed products such as wine, and being highly resistant to simulated gastrointestinal digestion. Thus the allergenic TLP from kiwi fruit is highly resistant to simulated gastrointestinal proteolysis, and the stability of TLPs to food processing is shown by the presence of allergenic grape TLPs surviving the vinification process and being found in wine. It is likely that the rigidity of the protein scaffold introduced by intramolecular disulfide bonds is responsible for the stability of allergens such as LTPs, and TLPs are probably reponsible for their stability to proteolysis. Similarly, the intramolecular disulfide bonds in the chitin-binding domain class I chitinases may confer stability, although the allergenic homolog from avocado, Pers a 1, is extensively degraded when subjected to simulated gastric fluid digestion. However, the resulting peptides, particularly those corresponding to the hevein-like domain, were clearly reactive both in vitro and in vivo.

Tree nuts and seeds

The major allergens of tree nuts and seeds include other members of the prolamin superfamily, the 2S albumins and the cupin seed globulins, both of which often function as a protein store in the seed. 2S albumins have been identified as important allergens in nuts, including walnut allergen (Jug r 1), almond, Brazil nut (Ber e 1), hazelnut and pistachio (Pis v 1) and in seeds such as oriental and yellow mustard (Bra j 1) and (Sin a 1), Ses i 1 and 2 from sesame, and the 2S albumin from sunflower seeds (SFA-8). These allergens seem to be highly potent and may well dominate allergic responses to many nuts and seeds. In addition to the 2S albumins, a second major group of allergens found in nuts and seeds are the 11S and 7S seed storage globulins that belong to the cupin superfamily. Seed storage protein allergens have been described in a variety of nuts and seeds, with both 11S and 7S seed storage globulins having been reported as allergens in hazelnut (Cor a 11 [7S globulin] and Cor a 9 [11S globulin]), cashew nut (Ana o 1 and Ana o 2) pistachio (Pis v 2 and Pis v 3), walnut (Jug r 2 and Jug r 4), and sesame seed (Ses i 1, Ses i 6). The 11S globulins have also been shown to be allergens in almond, also known as almond major protein (AMP) and mustard (Sin a 2). The close botanical relatedness of species such as cashew and pistachio and the high levels of homology between the major allergens in these tree nuts explain the cross-reactive nature of allergies to these nuts. There are suggestions that conformational epitopes exist in these proteins, which are also responsible for IgE cross-reactivity between allergens from species where homologies are weaker. However, it is difficult to distinguish between polysensitization and cross-reactivity.

In addition to the pollen–fruit cross-reactive allergy syndromes, it is emerging that Bet v 1 homologs in various nuts and seeds can cause similar allergies. These have been especially well documented for hazelnut, where an isoform, Cor a 1.04, has been identified which resembles Bet v 1 more closely than the allergenic Bet v 1 homolog from hazelnut pollen (Cor a 1.01). There are also reports of LTPs found in nuts and seeds triggering allergies similar to those observed in fruits such as peach, including LTP allergens from walnut (Jug r