

REVIEW

Immunogenic and Allergenic Potentials of Natural and Recombinant Innocuous Proteins

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A new aspect of protein immunogenic and allergenic properties has become important recently, when there is a higher chance that our immune system will be exposed to novel protein antigens and/or familiar protein antigens with an unprecedented high frequency and large amount. These proteins are innocuous, nontoxic, and noninvasive by themselves, and include various natural proteins from the environment and recombinant proteins from industry. The technical term allergenic has been used for such proteins and their abilities to induce specific IgE production and to cross-link IgE/FcεRI on the surface of mast cells and basophiles have been recognized. As for the environmental proteins, some physicochemical properties (solubility, stability, and permeability across a mucosal epithelium) of the proteins indirectly play important roles in their allergenic potential because they do not originate from invasive pathogens as vehicles. Indeed, several lines of experimental evidences have been accumulated indicating that all proteins are absorbed across mucosal epithelia by transcellular transport and/or through interstitial spaces among the epithelial cells but not at equal levels. Some animal models have been established for natural sensitization to some allergenic proteins by feeding or intragastric administration without an adjuvant and, in a few cases, some symptoms resembling human allergy and even anaphylaxis have been induced by oral challenge with the proteins. Sometimes, even to self-proteins, the immunogenic or allergenic potential is given by post-translational modifications and possibly by unknown structural/conformational alterations, when they are exogenous self-proteins, such as recombinant human proteins for drug use. Despite the accumulation of knowledge and the progress in analytical technology on protein allergenicity, it is still crucial to predict the allergenic potential of novel and unused proteins. However, some animal models are applicable for assessing the relative allergenic potential of processed proteins in comparison with that of native proteins in preclinical studies.

[Key words: allergens, food allergy, protein drug, protein absorption, animal model, genetically modified food (organisms)]

The immune systems of higher animals play a central role in body defense against a large variety of infectious microorganisms, such as viruses, bacteria, fungi, and parasites. Macromolecules, including proteins, polysaccharides, and complex lipids, expressed by infectious, or even noninfectious, microorganisms are recognized, attacked and memorized as foreign antigens by the immune system to prevent the multiplication of infecting microorganisms. Thus, the immunological aspects of proteins have long been investigated mainly from pathological and microbiological viewpoints in the medical and clinical fields.

Later, protein antigens recognized by the immune system without infection became to be considered in the immunological research field, even though they were innocuous by

themselves. Some of these are cell surface membrane proteins with genetic polymorphism, the major histocompatibility complex (MHC) molecules, which are recognized as non-self molecules and attacked as target antigens (alloantigens) in the rejection of tissue and organ transplantation between genetically different animals of the same species (1, 2). The others are a variety of protein antigens from our environment. These include proteins in food, house dust, tree (or grass) pollen, and other natural products, which are frequently present in our environment. These environmental proteins are derived mostly from nonpathogenic eukaryotes (plants and animals) and are principally innocuous by themselves and, therefore, it is not necessary for the immune system to respond to them. In some cases, however, the immune system responds to such innocuous proteins and involuntarily causes pathological damage to the host organisms own tissues and organs and occasionally serious sys-

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temic pathologies. The diseases caused by such undesired immune responses to innocuous antigens, mostly proteins, are referred to as allergies. The prevalence of allergy is not precisely known but is believed to have increased in recent decades (3, 4). In connection with the increase in the number of clinical cases of allergy, the immunological properties of proteins from a large variety of organisms, including plants, insects, fish, birds and mammals have become a topic of interest not only for clinical doctors but also for researchers, industrial producers and consumers.

Recently, the immunogenic and allergenic potentials of proteins have become a matter of special importance for a group of proteins produced using gene and cellular technologies. One is the protein produced by transgenic plants commercially handled in the market, referred to as GMO (5, 6), whereas the other one is a recombinant protein for clinical use, referred to as a biopharmaceutical or biological drug (7–9). With regard to food safety and public acceptance/understanding, the allergenic potential of transgene products, particularly novel proteins in food, such as GMO is of great importance. Among a variety of biopharmaceuticals, recombinant protein drugs, such as peptide hormones, cytokines, enzymes, and antibodies, are considered to be potential immunogens/allergens to human and domestic animals in some cases (7, 9), even though all of these biopharmaceuticals are self-proteins but expressed by cultured cells of some other species.

In this short review, we focus on providing information on the basis of recent research works, including our unpublished results, about the estimation and assessment of immunogenic and allergenic potentials of the food and environmental innocuous proteins and recombinant self-proteins used clinically. Regulatory issues and technical problems on the control and assessment of protein immunological potentials are also discussed.

DEFINITIONS OF IMMUNOGENICITY AND ALLERGENICITY

The term immunogenicity is used generally for the ability of proteins or any other molecules to stimulate the immune system and induce cellular and/or humoral immune responses, including antigen-specific lymphocyte proliferation/differentiation and antigen-specific antibody production. This definition was given, when immunological responses against invading microorganisms were seriously important in the prevention and therapy of infectious diseases. Therefore, in such infectious diseases, it is postulated that proteins derived from microorganisms are present in the body of the host, and the proteins are exposed directly to phagocytotic and/or antigen-presenting cells, the antigen receptors of B lymphocyte and secreted antibodies. On the other hand, in recent years, the immune responses against proteins from nonmicrobial and nonpathogenic organisms have become of research interest. These are the types of environmental proteins, which we are exposed to daily, such as those from food, drugs, house dust, and tree and grass pollens, and they are generally innocuous by themselves. When the immunogenicity of these environmental proteins is described and discussed, in addition to the ability to stim-

ulate the immune system and induce a response, some other properties of these proteins should be considered regarding their absorption, permeation or infiltration into the body across the mucosal epithelium and skin (epidermis). Such properties might be included in the immunogenicity in a broad sense for the proteins derived from noninfectious organisms. We sometimes use the term oral immunogenicity for the ability of proteins ingested orally, *e.g.*, food proteins, oral drug proteins and oral vaccines to induce an immune response. Particularly in such a case, when the oral route is used, some abilities/properties to resist digestive enzymes and to be recognized by gut-associated lymphoid organs (GALT) would largely affect their immunogenicity in addition to their absorption into the body across the intestinal mucosal epithelium. Thus, such properties as stability and permeability (solubility) might be included in the general definition of oral immunogenicity.

The definition term of allergenicity is basically identical to that of immunogenicity, but the use of allergenicity is restricted to cases where the immunological responses induced by the protein lead to the provocation of pathological symptoms, such as allergic disorders. Allergic disorders have a large diversity in both immunological mechanisms and pathological symptoms, and are categorized generally into four types. The type I allergy, also referred to as the immediate type or the IgE-mediated type, has been most extensively investigated and its molecular and cellular mechanisms are relatively well understood. Therefore, we focus on type I allergy to describe allergenicity in this review.

In general, the provocation of allergic disorders is further divided particularly by professional clinical doctors and researchers into two distinct steps named sensitization and elicitation. Sensitization, which is a type of immunization, refers to the sequential immunological events including the antigen stimulation in cells responsible for antigen-specific immunological responses, termed adaptive or acquired immune response, the induction of antigen-specific IgE class antibody production, and the subsequent IgE binding to its specific receptors (FcεRI and II) expressed on the surface of mast cells and basophiles in various tissues. The last IgE-binding event on mast cells is sometimes called mast-cell sensitization. The elicitation implies the activation and degranulation of sensitized mast cells, that is, the release from the mast cells of both preformed and newly formed mediators including histamine, prostaglandins, and leukotrienes, and the subsequent physiological responses and clinical symptoms in a variety of cells, tissues and organs. Thus, the allergenicity of proteins depends on both its abilities to sensitize mast cells and to elicit clinical symptoms prefaced by mast cell activation/degranulation. However, the definition of protein allergenicity based on these two abilities is not necessarily applicable to all cases. For example, these are cases in which mast cells are sensitized with IgE antibodies raised against a certain protein and subsequently elicited by other unrelated proteins sharing some common cross-reactive epitopes (with immunological cross-reactivity). Therefore, it is possible that a protein possesses the sensitization ability but not that of elicitation, and vice versa, as described later. Thus, both sensitization and elicitation have to be considered when the allergenic potential of proteins is

described.

**PROTEIN ABSORPTION/PERMEATION
ACROSS EPITHELIUM:
PART OF IMMUNOGENICITY AND
ALLERGENICITY OF ENVIRONMENTAL PROTEINS**

The ability of environmental proteins to enter into the body could be regarded, at least in some cases, as a factor in the immunogenicity and/or allergenicity of the protein, because how much and from where proteins enter into the body across the epidermis or mucosal epithelia affect the recognition of and response to the protein by the immune system as well as the elicitation of an allergic reaction and symptoms (10). In spite of such importance, the ability of proteins to act as immunogens and allergens that enter into the body has not been intensively investigated until recently. Experimental and clinical studies have so far mainly shown the absorption or uptake of orally ingested or infused proteins from the intestinal lumen into the blood stream and lymph (11–14), and that of inhalant proteins from the respiratory or olfactory organ, *e.g.*, nasal cavity (12, 15), whereas almost no studies have been reported on those from ophthalmic mucosal epithelia (16). Table 1 shows the summary of the data obtained from these experiments under *in vivo* and *in situ* conditions. The approximate plasma concentrations of the absorbed proteins appeared to be similar among animal species including humans, that is, 1–1000 ng/ml. The time periods required for the plasma concentration to reach maximum are 15–20 min for egg ovalbumin (OVA) in mice (Matsubara, T. and Matsuda, T., manuscript in preparation), 60–120 min for OVA in rats (12), and 30–90 min for bromelain in humans (13). A characteristic time-dependency in the protein absorption in 19 healthy individuals was also reported. The night intake of bromelain resulted in

a plasma concentration about 5-fold that for the daytime intake (13). Many studies on protein absorption using *in vitro* systems of epithelial-cell monolayer culture are not addressed in this review.

The absorption of proteins across the intestinal epithelium has been demonstrated in experimental animals such as rodents, as well as healthy human subjects. In these experiments, some well-characterized proteins, such as OVA (12) and egg lysozyme (18), have been used as model proteins. In some cases, a soybean allergen (19), lactoferrin (20) and bacterial protease (21) as potential oral drug proteins were also analyzed. It is worth noting that all of these proteins are soluble in physiological solutions. It has been agreed that food protein's solubility (to cross the gut mucosal barrier and epithelium) and stability (to resist enzymatic degradation) are the important characteristics as food allergens among the intrinsic protein properties (22). However, these characteristics are not solely sufficient but are vital factors. It is difficult to directly compare the data on protein absorption/uptake reported in these previous studies because of the diversity in experimental conditions used in these studies. Therefore, intrinsic factors and properties responsible for protein permeability across the epithelium are unpredictable at this stage based on the limited data available. Nevertheless, a few studies suggested that an electrical charge in protein affects its absorption from intestinal lumen (14, 17). Some positively charged proteins (higher pI proteins), such as lysozyme, bromelain, and pancreatopeptidase E, have been shown to electrostatically interact with the negatively charged surface of epithelial cells, and such electrical attraction between the protein and intestinal epithelium is suggested to increase the probability of the protein being nearby the cell surface and hence protein's absorption rate across intestinal epithelium (13). Anionization and cationization of lysozyme by chemical modification, which

TABLE 1. Protein absorption across mucosal epithelia

Protein and administration route	Serum/plasma concentration	Test subject	Reference	Year
1. Digestive and absorptive epithelium: stomach and small intestine				
Human (healthy subjects)				
Egg lysozyme (30, 90 mg, Neuzym Eisai), oral	1.7 ng/ml, 37 ng/ml after fasting	22 male, 20–45 years	17	2002
Pineapple bromelain (200 mg, enteric-coated tablets), oral	5 ng/ml, an average of 10.8 microgram in plasma	15 male, 18–45 years in the 3- to 51-h period, complex with proteinase inhibitors	13	1997
Cow's milk β -lactoglobulin (10 ml milk/kg body), oral	3–5 ng/ml in serum for 120 min	8 male/female, 18–22 years	11	1993
Murine (whole body or <i>in situ</i> closed loop)				
Soybean allergen (Gly mBd 30K), 500–2000 mg/kg, oral	77–391 ng/ml, 30 min later	24-d old mice	19	2005
Lactoferrin, 1 g/kg, infusion into the duodenal lumen	Via lymphatic pathway, not via portal	Adult rats	23	2004
Egg lysozyme, closed loop, perfusion of 0.35 μ M	80 pmol/ml (1.1 μ g/ml), 30 min later	Adult rats	18	2004
Egg lysozyme, SOD, 1 and 10 mg/kg, closed loop (jejunum)	0.002% of dose, 60 min later	Adult male rats	14	2002
Egg ovalbumin, 10 and 50 mg /rat, oral	2–30 ng/ml, 60 min later	Adult rats	12	1996
Serratia protease, 5 mg/kg, oral	(equivalent to 4 ng/ml), complex with α -2M	Adult rats	21	1982
2. Respiratory/olfactory epithelium: nasal cavity				
Murine				
IGF-I, 5 nmol/rat, intranasal	5 nM, 6 h later	Adult rats	15	2004
Egg ovalbumin, 2.5 mg/rat, intranasal	50 ng/ml, 120 min later	Adult rats	12	1996

did not affect its enzymatic activity, markedly decreased and increased the protein absorption rate, respectively (14). Another enzyme, superoxide dismutase (SOD), which is anionic under neutral pH, showed an absorption rate lower than lysozyme, and the cationization of SOD also increased the absorption rate (14). Thus, the intestinal absorption of a protein is, at least partially, determined by its electrical charge.

Lactoferrin, a glycoprotein present in milk and other secretions, has been reported to be absorbed by receptor-mediated transcytosis in the intestinal epithelium of rats (23) and pigs (20). Another absorption system using receptor-mediated transepithelial transport has been reported on CD23, the low-affinity receptor for IgE (FcεRII), which is expressed inducibly by sensitization on the epithelial cell microvillous membrane of rodents and humans (24, 25). The sensitization of animals with a protein induced FcεRII expression on enterocytes and specific IgE production, and enhanced the uptake of FcεRII/IgE/antigen triple complex into enterocytes, resulting in the transepithelial transport of the protein (26). Therefore, it could be said that proteins with a higher immunogenic (IgE producing) potential have a higher transepithelial permeability.

The protein absorption and transepithelial transport in the olfactory organ has been intensively investigated from the viewpoint of protein-drug delivery into the brain through olfactory nerve cells. An electron microscopic study in 1986 demonstrated that intranasally administered horseradish peroxidase (HRP) migrates through open intercellular clefts of the olfactory epithelium to the olfactory bulbs of mice, rats and squirrel monkeys within minutes of administration (27). Afterwards, several studies on pharmaceutical proteins, such as nerve growth factor (NGF) (28), fibroblast growth factor-2 (FGF-2) (29), insulin (30), and insulin-like growth factor I (IGF-I) (15), have demonstrated that after intranasal administration these proteins were absorbed and transported to brain tissues or cerebrospinal fluid. More quantitative analyses of OVA, which had been administered into nasal cavity of rats (2.5 mg/rat), revealed that the plasma concentration of OVA increased linearly for 60 min after administration and reached the maximum (about 50 ng/ml) a further 60 min later (12). Interestingly, such a plasma concentration of OVA after nasal administration was much higher than that after oral administration (50 mg/rat). In the case of protein absorption via the nasal cavity, the transepithelial transport of a protein would be affected simply by its intrinsic physical and/or chemical properties but not by its stability against proteases and acid, which would be important factors affecting the final transepithelial transport of orally ad-

ministered proteins. Hence, transepithelial transport in the nasal cavity might be a good model system for the evaluation of permeability across epithelium for proteins.

ANIMAL MODELS FOR STUDIES ON IMMUNOGENICITY AND ALLERGENICITY OF ORAL PROTEIN ANTIGENS

When proteins are injected directly into the body (i.v., intravenously; s.c., subcutaneously; or i.p., intraperitoneally), the assessment of protein immunogenicity would be simple, that is, all proteins except for self-proteins are basically immunogenic. However, it is much more complicated and difficult to assess the immunogenicity and allergenicity of proteins which are ingested as food, because such oral immunogenicity of proteins is affected by several factors including enzymatic degradation in the gut, transepithelial transport from gut lumen into body fluids, and immunological tolerance to oral antigens (31). The normal immune response to dietary proteins is an increase in secretory IgA released into intestinal lumen and, at the same time, the active suppression of the systemic immune response (oral tolerance). Presumably, genetically predisposed human individuals or animal strains respond to innocuous food proteins in an abnormal hypersensitive manner. Therefore, only a limited number of animal models for the studies on oral protein allergenicity have been reported so far (22, 32), and even these models could not necessarily be utilized for completely assessing the oral allergenic potential of a variety of proteins. The intensity of the IgE antibody response and the breakdown of the state of oral tolerance are affected generally by many factors attributed to both proteins and animal immune systems. The parameters to be considered in animal model experiments were well described by Helm (22), and are summarized in Table 2.

In the case of mice, a variety of inbred and congenic strains have been established and used for immunological research. Our preliminary experiments on the oral sensitization by food proteins using various inbred strains of mice suggested a strong strain dependency in the antibody response to a particular protein and the isotype (only IgG or both IgG and IgE) (Matsuda, T., unpublished results). Several inbred strains (A/J, Balb/c, C3H/HeJ, C57BL/6, C57BL/10, DBA/2) and *H-2* congenic strains (B10.A, B10.D2, B10.BR) of mice were simply fed a purified experimental diet containing 20% (w/w) protein as a nutrient, which consisted of a single food protein (milk casein, whey protein isolate, egg white, soybean protein isolate, or rice protein concentrate) for 2 to 4 weeks, and the serum IgG

TABLE 2. Parameters responsible for animal sensitization model

1. The dose/concentration of the protein (high doses and very low dose often induce tolerance)
2. The route of protein administration (feeding or gavage is recommended)
3. The duration of allergen exposure (administration periods and frequency)
4. The age of the animal (neonate, adolescent, adult)
5. A genetic predisposition (high and low IgE responders and strain MHCs)
6. The use of adjuvant (natural sensitization is desirable, and cholera toxin, <i>Bordetella pertussis</i> , and carrageenan are known IgE-selective adjuvants)
7. The isotype specificity response (both IgG1 and IgE are anaphylactic antibodies in mice and guinea pig, IgG2a and IgE in rats, IgE in dogs)
8. The Th1/Th2 regulation/polarization (mice have very delineated Th1/Th2 polarization, whereas in the human polarization it is not as discrete)

TABLE 3. High responder strains of mice to oral protein antigens

Mouse strain (<i>H-2</i> haplotype)	Protein antigen	
	IgE response to	IgG response to
B10.A (<i>k/d</i>)	Egg lysozyme	Egg lysozyme
	Milk casein	Milk casein
B10.BR (<i>k</i>)	Egg lysozyme Milk casein	Egg ovomucoid
		Egg ovotransferrin
		Rice globulin
		Egg lysozyme
		Milk casein
B10.D2 (<i>d</i>)	Egg ovotransferrin	Egg ovomucoid
		Egg ovotransferrin
DBA/2 (<i>d</i>)	Milk casein	Milk casein
		Milk casein
A/J (<i>k/d</i>)		Egg ovomucoid
		Egg ovotransferrin

and IgE responses to each purified protein contained in the experimental diet were analyzed. As summarized in Table 3, the particular combinations of each protein with the mouse strain made the immune system respond to that dietary protein without an adjuvant, whereas the other combinations did not. It should be noted that under these experimental conditions the major or minor constituent proteins are not necessarily the causative immunogen for the IgG and IgE responses and that the isotypes of the immunoglobulin raised against the oral protein antigens were both IgG and IgE, or only IgG, but the IgE response without IgG was not observed. Thus, the induction of the IgE response to dietary proteins appeared to be more difficult to achieve than that of IgG by natural sensitization in mice.

Some mouse models for specific food allergies such as cow's milk (33, 34) and peanut (35) allergies have been reported. DBA/2 mice gave not only a serum IgE response (Table 3) but also a predominant Th2-type T-cell response against cow's milk casein in the diet (33), suggesting a natural sensitization by casein feeding (33, 34). On the other hand, with the help of cholera toxin as an adjuvant, the oral sensitization of C3H/HeJ mice to cow's milk was achieved to the level where pathological hypersensitive symptoms could be induced by the subsequent re-exposure (intragastric challenge) to cow's milk (34). This C3H/HeJ model using cholera toxin as an adjuvant was reported to be applicable also to the peanut allergy model, and in that study fatal or near-fatal anaphylaxis was induced in 10–20% of the sensitized mice by the oral challenge of 10 mg of crude peanut extract (35). Mouse models for food-dependent exercise-induced anaphylaxis (FDEIA) have recently been reported on egg lysozyme (36) and wheat glutenin and gliadin (37).

The Brown Norway (BN) strain of rats is known to be a high immunoglobulin (particularly IgE) responder strain (38) and, thus, have been used for the study of oral sensitization to food proteins, such as administration through the diet or by gavage dosing either in the presence or absence of an adjuvant. A typical successful protocol for sensitization used in one of these studies was the daily intragastric administration of 1 mg OVA for 42 consecutive days, which induced OVA-specific IgG and IgE responses in the majority of rats (80% or more) (39). The successful oral sensitiza-

tion of BN rats has been reported also with proteins from chicken egg white (40), cow's milk (40), and soybean protein (41).

The experimental models using some other animal species, such as dogs, swines, and guinea pigs, were described in the reviews of Helm (22) and Kimber *et al.* (32).

IMMUNOLOGICAL RESPONSES AGAINST RECOMBINANT SELF PROTEINS: BIOPHARMACEUTICALS

Through recent development of recombinant DNA technology and cell engineering the large-scale production of human proteins has become possible and, at the same time, a novel immunological problem has been elicited. A variety of human proteins, such as growth factors, cytokines, peptide hormones, and immunoglobulins (antibodies), have been produced as recombinant proteins expressed in bacteria, yeast, and insect and mammalian cells, and even in transgenic livestock, and widely used for clinical therapeutics (7, 42, 43). Primarily, self-proteins are not recognized by the immune system and thus nonimmunogenic. However, it is well known that several post-translational modifications of proteins occur in the host cell biosynthesis system, and that quantity and quality of such modifications differ depending on the host organism. A typical example of such a problematic modification is glycosylation (8). Yeast, fungi, higher plant, and insect and mammalian cells, which are often used as host organisms for the production of recombinant proteins, synthesize characteristic structures of sugar chains as a portion of glycoproteins and glycolipids. Prokaryotic organisms have long been believed not to synthesize glycoproteins and it is now evident that they do generate glycoproteins and glycolipids (44, 45). Some pathogenic bacteria can even synthesize glycoprotein sugar chains that resemble those of mammals (46, 47). On the other hand, among mammals, only humans, apes, and Old World monkeys do not synthesize the Gal α 1-3Gal structure at the non-reducing end of sugar chains (48). Thus, the fine structure of sugar chains on recombinant glycoproteins differ from authentic ones synthesized in human and animal bodies. Such differential post-translational modification could be a cause of immunological xenogeneity of self-proteins.

Recombinant proteins with Asn-X-Thr/Ser expressed in plants and insect cells often undergo *N*-glycosylation. The *N*-linked sugar chains which are synthesized and added to proteins in plant and insect cells, but not in mammalian cells, often contain β 1-2 linked xylose (X) and/or α 1-3 linked fucose (F) residues attached to the glycan core structure (Man3GlcNAc2) (49, 50). Such xylose/fucose (X/F)-containing sugar chains act as haptenic antigens in mammalian immune systems, because mammalian glycoproteins and glycolipids have no such X/F-containing sugar chains. This X/F-containing sugar chain is well known to be an epitope for IgE antibodies, which are often found in the serum of patients allergic to tree and grass pollens, fruits and vegetables (51–55). Strictly speaking, no true immunogen(s), which induces an antibody response specific for this carbohydrate epitope, is known, because the sugar-specific antibodies cross-react with a large variety of glycoproteins

derived from plants and insects, and in many cases no antibodies specific for the polypeptide portion of the allergenic glycoproteins are present in the patients' serum. Recombinant self-proteins with the X/F-containing sugar chain would be recognized and picked up by the pre-existing antibodies in the human serum, and might be treated and processed as non-self proteins by the immune system (56). It is uncertain whether the self-proteins become immunogenic when such X/F-containing sugar chains are added (57).

It has long been a controversial issue that glycoproteins with multiple X/F-containing sugar chains can induce allergic symptoms, that is, degranulation of mast cells or basophiles sensitized with the sugar-specific IgE antibodies (54, 58). A recent clinical study demonstrated that approximately one third of the patients allergic to tomato had the serum IgE antibodies, which could induce degranulation of basophiles through binding to unrelated glycoproteins with multiple X/F-containing sugar chains (59). Also in our recent research on a murine model, a glycoprotein with multiple X/F-sugar chains induced degranulation of rat basophilic leukemia cells (RBL-2H3) sensitized with mouse IgE antibodies specific for the X/F-containing sugar chain (Hino, S. and Matsuda, T., manuscript in preparation). Therefore, recombinant glycoproteins, even self-proteins, produced in plant expression systems, are potentially allergenic.

The conversion of self-proteins to immunogenic variants (altered form) by unknown mechanisms including post-translational modifications other than glycosylation have been reported on several recombinant human proteins for pharmaceutical use, such as insulin, interferon, and erythropoietin. In these cases, the production of antibodies specific for the recombinant self-protein was observed in the serum of patients treated long term with the protein drug, resulting in the lack of drug efficacy or even hypersensitive reaction to the drug (7, 9, 60). The possible mechanisms for the unexpected antibody response to exogenous self-proteins have been considered to be some enzymatic and chemical modifications of proteins (61), such as amino acid modifications (*e.g.*, the deamidation of Asn, isomerization of Asp, and a deimination of Arg [citrullination]), and conformational alterations including the aggregation of the protein (62). Some mechanisms in the immune system would also be responsible such as the genetic predisposition to form autoantibodies, and the previous exposure of an individual immune system to identical or similar proteins.

Monoclonal antibodies, which are identical or similar to human antibodies (human, humanized and chimeric antibodies in order of the identity level), have been manufactured for therapeutic use in malignancies and some other diseases (9). The humanized and chimeric antibodies are not self-proteins and, therefore, the production of anti-antibodies (antibodies against the monoclonal antibodies) would be caused if these antibodies were used repeatedly. Furthermore, even human antibodies become immunogenic in the human immune system when they are monoclonal (63). Immunoglobulins consist of both variable and constant regions, and the amino acid sequences of the variable region of the serum antibodies have great diversity (polyclonal). By contrast, monoclonal antibodies are homogeneous in their whole amino acid sequence and, consequently, have a com-

mon and identical variable region sequence. The frequent administration of such a monoclonal antibody might result in the unusually high exposure of an epitope in the variable region to the immune system, and accordingly induce the response of anti-idiotypic antibodies (anti-variable region antibodies) (64). Thus, monoclonal antibodies have a unique and specific property as a self-antigen.

PREDICTION OF PROTEIN ALLERGENICITY: ASSESSMENT OF GENETICALLY MODIFIED CROPS AND INDUSTRIALLY PROCESSED FOODS

Although an accurate assessment of the allergenic potential of a certain protein or its processed products is not easy even with the use of latest knowledge in immunology and front-line technology, the importance of and social needs for the allergenicity assessment have recently been increasing particularly for the food-industry and bio-industries from viewpoints of safety and quality of the industrially processed products. In Japan, the number of reported clinical cases on food allergy has recently increased and the regulation of the labeling of potential allergens in food products started in 2003. A questionnaire-based survey of about 20,000 subjects conducted by the Japanese government in 1998 reported that about 9% of infants (3 years old), 6–8% of children (7–14 years old), and 9% of adults experienced some subjective food-allergic reactions, and the frequent causative foods for such subjective allergic reactions were eggs, milk, fish, and shellfish. Afterwards, five foods (wheat, buckwheat, eggs, milk, and peanuts) were included in the list of potentially allergenic foods that should be labeled in processed food products. These allergenic foods are those that have ever been identified and reported by clinical doctors in hospitals to be a causative food of severe allergic symptoms. In this case, the assessment of the allergenic potential of protein(s) in the causative food would be reasonable because that protein(s) has sensitized some individuals and elicited a severe allergic reaction. However, there are some difficulties in the assessment for novel proteins that have never been ingested as food. The word “novel” indicates not only proteins of nonfood origin but also food proteins modified or processed by novel technologies. The former include novel bacterial and fungal enzymes used for food processing and bacterial insecticidal proteins used for genetic modification of commercially valuable plants, whereas the latter include some proteins modified physically, chemically, or enzymatically to reduce the allergenic potential of food known to be allergenic.

In the case of novel proteins, regardless of whether they are natural or recombinant, originated from non-self organisms, which have never been used and ingested as food, we have no reliable methods to determine their allergenic potential (6, 65). The only method is the oral administration or ingestion test by a large number of volunteers over a considerable period. However, sensitization studies would be impossible or difficult for ethical and efficacy reasons. As introduced in a few previous review papers (5, 6), the allergenic potential of such novel proteins is now assessed by comparison of several properties of the questionable protein

with those of known allergenic proteins. Such properties include digestibility, sequence similarity to known allergenic proteins, and the *in vitro* and *in vivo* reactivity with IgE antibodies in the serum originated from patients allergic to the known allergen with structural similarity to the questionable protein. This assessment seems reasonable for determining whether the novel protein elicits allergic symptoms in the already sensitized patients, and this is actually the best that we can do now. However, the ability of a novel protein to sensitize someone could not be estimated by this assessment on the basis of the known allergens and the antibodies to them.

Furthermore, even on the ability to elicit allergic symptoms starting from the mast cell activation by the protein/IgE binding, the assessment based simply on the sequence similarity may not be correct in some cases. For example that is the case that the sequence similarity of the novel protein to a known allergen is present only in a limited region, that is, only one sequence consisting of ten amino acid residues is identical between the novel protein and a known allergen. First, the possibility is rather low that IgE-epitope of the known allergen is located accidentally in that sequence of ten residues. Secondly, even if that identical sequence indeed contains an IgE-epitope, proteins with only a single IgE-epitope could not elicit the mast cell activation followed by degranulation because the cross-linking of at least two IgE molecules/FcεRI on the mast cell surface by a multivalent allergen is the minimum requirement for the mast cell activation. It is theoretically possible that a novel protein shares one or a few identical or similar sequences with a known allergen. Even in such cases, however, we could not conclude that that the novel protein is a clinically relevant cross-reactive allergen and will elicit allergic symptoms when ingested. Therefore, the *in vitro* degranulation assay would be required for the final decision by using sensitized basophils derived from patients sensitive to the known allergen with similar/identical sequence(s).

An example of a kind of novel processed protein is the insolubilized egg white proteins prepared by severe heat treatment followed by complete washing. In collaboration with a food company, we investigated the oral immunogenicity and allergenicity of the heat-denatured and insolubilized egg white proteins using the oral sensitization model of B10.A mice/egg white proteins described above. The working hypothesis was that the lack of solubility, a possible minimum requirement for food allergens, might reduce or eliminate the oral allergenicity of egg white. Chicken egg white was processed industrially by boiling to denature the egg white proteins, and the resultant coagulates were minced and washed with water to remove any remaining soluble proteins, resulting in the production of novel insoluble egg white aggregates. Feeding B10.A mice with the insoluble-egg-white-based diet induced no antibody (IgG, IgE) response to egg white proteins, either denatured or native ones, whereas that of a spray-dried egg white preparation did (Ishii, T. and Matsuda, T., unpublished results). According to this positive result in the mouse model, a clinical oral challenge test was performed by Urisu *et al.* (66). The placebo-controlled oral challenge of the insoluble egg white was done for the patients with positive reaction to the oral

challenge of raw egg white. All but one of the 32 egg-allergic patients were negative to the challenge with the insoluble egg white, suggesting that allergenic potential of the processed insoluble egg white was quite low (but not zero). Thus, some mouse models described above are reasonable and useful for assessing the relative allergenic potential of a certain processed food proteins compared with that of its native proteins, as a pilot study preceding clinical trials for patients.

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