Food and Agriculture Organization
of the United Nations

World Health Organization

Biotech 01/10

Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology

**Headquarters of the Food and Agriculture Organization of the United Nations (FAO)
German Room (C-269)
Viale delle Terme di Caracalla, 00100 Rome, Italy
22 – 25 January 2001**

Topic 8: Animal Model for Allergenicity Assessment

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Introduction

The possibilities of modern biotechnology has resulted in the introduction new generations of food and food ingredients and this process is expected to further continue in the near future. Especially, the development and market introduction of genetically engineered food crops has gained a lot of attention in the past few years. For these novel foods a toxicological evaluation and risk assessment are in most cases major items in their safety evaluation. Because some proteins in our food are responsible for the development of food allergy, the potential allergenicity of genetically engineered crops, mostly containing new proteins from non-food origin, has become an important issue of their safety evaluation. There is, however, no universal, reliable and relevant test available to evaluate the allergenic potency of food products and a case-by-case approach is suggested (Wal, 1999). The best known allergy assessment approach was jointly developed by the International Biotechnology Council and the ILSI Allergy and Immunology Institute (Metcalfe et al., 1996), in which the origin of the gene plays a central role. It is recommended in the so called IFBC/ILSI decision tree, which was based on the at that moment available data, that in case of transgenic proteins derived from non-food sources a careful step-wise process should be followed paying attention to several factors, like amino acid homology comparisons and various physico-chemical properties such as heat and digestive stability of the protein. For transgenic products derived from known allergenic foods several *in vitro* and *in vivo* test are available that will result in a conclusive assessment of its potential allergenicity.

It should be indicated that the endpoint of the decision tree was either that the product could be marketed or should be labelled.

Unfortunately, from the introduction of this decision tree it was evident that if the protein of interest does not have a history of dietary exposure or has an unknown history in terms of allergenicity, the tests indicated in the decision tree would not be conclusive with respect to the allergenic potential of the protein. It is clear that in case negative predictions are observed for all recommended tests there is a low probability that the protein is a significant food allergen, but still no full assurance can be given with respect to the sensitizing potential of the protein studied. However, especially if a positive prediction is observed for one of the listed parameters, like for instance with respect to the actual digestive stability observed with Cry9C in the Starlink corn of Aventis (EPA, 2000), further testing is warranted. These additional tests should preferably be focussed on the prediction of the sensitizing potential of the novel protein. An universal, reliable and relevant *in vitro* or *in vivo* test to study the sensitizing potential of a new protein is, however, not available. As different factors are known to influence the sensitization process in susceptible individuals and moreover the immune system is a very complex system, in which different cell types and mediators play important roles, it is not expected that this process can be studied *in vitro*. The most direct approach to determine the sensitizing potential of proteins is considered to be the development and validation of a widely accepted animal model. An important advantage of animal models may turn out to be the possibility to obtain information on the potential allergenicity of the new proteins in relation to information on its potency relative to well known allergens. In this paper some new animal models will be introduced that have become available in literature and are currently in development for testing of the allergenicity of foods or food proteins. In particular a promising model for food allergy research, developed in Brown Norway rats, will be presented. The results obtained from the BN rat studies demonstrate that they can be sensitized orally to the various allergenic food proteins tested, resulting in significant antigen-specific IgE responses, without the use of adjuvants. Upon an oral challenge in previously

sensitized animals also local and systemic immune-mediated effects could be observed like an increased gastro-intestinal permeability and decreased breathing frequency and blood pressure.

Animal models in allergenicity research of food proteins

As the various parameters currently used in the so called left arm of the ILSI/IFBC decision tree are not conclusive with respect to the sensitising potential of new proteins and no possibilities are available to assess the sensitising activity of new proteins involving human subjects, animal models suitable for research on the allergenicity of food proteins are considered to be of major importance. The need of accepted animal models was already clear at the time that the ILSI/IFBC decision tree was proposed (Taylor et al.,1996). Despite increasing research efforts in recent years to develop suitable animal models that mimic the induction of an IgE antibody response as the most remarkable marker of allergic sensitisation, and the induction of symptoms upon a challenge reaction like observed in human allergic patients, no validated and widely accepted animal model is still available. Also in the past several attempts have been made, however these efforts did not result in structured approaches aimed at the development of well validated enteral allergenicity models. The unscheduled dietary pre-exposure of the test animals or their parenteral generations to the protein under investigation may have been one of the major factors that has negatively affected the results of many oral sensitisation studies. Moreover, it was also known that oral exposure to food allergens easily resulted in induction of immunological tolerance in rodents (Stokes et al.,1983; Steinmann et al., 1990). For food allergy research, three rodent species have frequently be used, viz. guinea pigs, mice and rats. Nowadays, also other species like the dog are used (Ermel et al., 1997). In some of these experimental systems it was found that the stimulation of a persistent and vigorous allergen-specific IgE response required the co-exposure of the animals to adjuvants. For evaluation of the intrinsic potential of new proteins to induce an allergic sensitisation the presence of adjuvants is, however, not wanted. For oral sensitisation studies to food proteins guinea pigs have frequently been used (Devey et al., 1976; Piacentini et al., 1994) and found to be very sensitive. Using especially Active Systemic Anaphylaxis (ASA) study parameters in combination with Passive Cutaneous Anaphylaxis (PCA) testing guinea pigs are still used for allergenicity testing of amongst others hypo-allergenic infant formulas or other formulas based on modified (hypoallergenic) proteins. However, the significant differences in the immunophysiology of guinea pigs when compared with other species (no IgE as cytophylic antibodies), the limited knowledge of their immune system, the lack of tools to study its immune system, and the questionable specifity in allergic sensitization are the major draw backs for the further use of guinea pigs in food allergy research.

In the past oral protein administration studies in mice (diet/gavage/drinking water) without using adjuvants easily resulted in tolerance induction (Strobel et al., 1984; Mowat, 1987). More recent studies in mice, using repeated enteral protein administration in combination with adjuvants, show that immune priming or sensitization can be achieved (Challacombe et al., 1980; Strobel et al., 1986; Li et al., 1999; Li et al., 2000). Although the sensitisation protocols of the mice in the two studies of Li et al. were different he could orally induce allergic sensitisation of the mice to cow's milk (Li et al., 1999) and to peanut proteins (Li et al., 2000) using cholera toxin as oral adjuvant. In studies of Ito et al.(1997) oral immunisation of mice by feeding casein or ovalbumin as a constituent in the diet without adjuvant administration only resulted in allergic sensitisation to casein. Also the intraperitoneal route of exposure is studied in mice to assess the potential allergenicity of new proteins and these studies are focussed on both the antigenicity and allergenicity of these test proteins (Hilton et al.,1994, 1997, Dearman et al., 2000). The studies of Dearman et al.(2000) using ovalbumin (allergenic protein) and bovine serum albumin (limited

allergenic protein) indicate, that characteristic antibody (IgG and IgE) isotype profiles can be provoked by proteins which may be associated with variable allergenic activity.

Also in rats the oral sensitization to food proteins has been studied by administration through the diet or by gavage-dosing either in the presence (Bazin et al., 1976; Atkinson et al., 1996) or absence (Knippels et al., 1998a,b; 1999a,b; 2000) of an adjuvant. There may be some advantages to select the rat as a species for oral allergy research as the rat is the commonly used species in toxicity testing, so the oral sensitizing properties of a protein can be evaluated in perspective to the other information obtained on the health state, and there is a rather broad knowledge on the rat immune system, and many tools are available for immune related studies. As considerable variations exist between the different approaches followed in the different species there is an urgent need for the development of (a) predictive, validated and widely accepted animal model(s) to identify the potential allergenicity of novel proteins.

An ideal animal model: what are the criteria?

Despite the fact that animal models can provide important information on fundamental questions concerning sensitization and mechanistic research on (food) allergy, it is obvious that well-validated models do not exist yet to assess the allergenic potential of specific proteins. From the studies presented in the previous paragraph it is clear, however, that several groups are active nowadays to develop new approaches in animal studies for testing the allergenicity of (new) foods or food proteins. To become widely accepted such animal models should satisfy several important criteria as was also indicated by Taylor et al. (1996). The most important criteria are considered to be:

- sensitization and challenge should preferable be orally; it is evident that natural barriers such as the gastro-intestinal acid denaturation and enzymatic digestion and the mucosal/epithelial layers, which are all known to prevent, reduce or in any other way influence the allergenicity of proteins should be taken into account (Atkinson et al., 1996; Pauwels et al., 1979; Strobel et al., 1984; Turner et al., 1990)
- preferably no use of adjuvants: although in real life situations adjuvants may play a role in the sensitization process it is preferred to assess the intrinsic allergenicity of the protein itself
- the test animal should produce a significant amount of IgE and or other Th2 specific antibody(sub)classes
- the test animal should tolerate most food proteins
- upon a challenge with the allergen clinical reactions with respect to organ sensitivities should be reflected similar to those seen in humans
- antibody responses should be directed to similar proteins in the allergenic food as found in patient sera
- the model should be relatively easy to conduct and reproducible both in time and in different laboratories.

The Brown Norway rat food allergy model

Despite the fact that immunological tolerance induction was reported to occur in rats upon oral antigen feeding (Steinmann et al., 1990), it was not observed to be the general response. Therefore, also because of the known limitations of guinea pigs and mice, we considered the rat

to be the (most) promising species for the development of an oral feeding protocol. Our studies to orally sensitize rats to food proteins were started with Brown Norway (BN) rats. This high-immunoglobulin (particularly IgE) responder rat strain was selected as, to a certain degree, they resembles the atopic constitution of humans with respect to their genetic predisposition to react with an overproduction of IgE to antigens. Ovalbumin (OVA), a well defined chicken allergen, was used as a model allergen in our first studies. In these first studies not only different sensitization protocols were used, but OVA was also administered either via the drinking water or by gavage. Remarkably, successful sensitization was only observed in case of daily oral gavage dosing of the animals (Knippels et al., 1998a). Subsequent comparative sensitization studies using different strains of rats revealed that indeed the BN rat is the preferable strain of rats for oral sensitization studies. From the results of these studies it was evident that upon oral exposure of Wistar, PVG, Hooded Lister, and BN rats to OVA, only the BN rats developed OVA-specific IgE antibodies confirming the choice of the BN rat (Knippels et al., 1999a). The selectivity of the antibody responses in the BN rat (IgE) to hen's egg and milk proteins was studied with serum of sensitized BN rats and that of allergic patients to these allergenic foods, and found to be directed to the same proteins in these allergenic foods (Knippels et al., 2000). From our sensitization studies with soy it was clearly demonstrated that exposure of the parental generation to the antigen under investigation will influence the outcome of sensitization studies with the offspring (Knippels et al., 1998b). Therefore, when oral sensitization studies with proteins are performed one has to ensure that at least two generations of animals have to be bred on a diet free of the antigen under investigation to get immunological naïve animals. To further characterize the BN rat food allergy model immune-mediated effects were studied upon oral challenges showing effects on breathing frequency, gastro-intestinal permeability and on blood pressure (Knippels et al., 1999b). All together, these observations suggest that this BN rat model might provide a suitable animal model to study the allergenicity of food proteins, although further validation of the model is needed.

MATERIALS AND METHODS

For detailed information on the rats and the materials used in the various sensitization studies, and the procedures used to conduct the immunological measurements [total and antigen-specific IgG/IgE antibodies, Passive Cuteneous Anaphylaxis (PCA) testing for measurement of reagenic antibodies, measurement of Cell Mediated Immunity parameters], and to measure immune-mediated reactions in sensitized rats (gut permeability, blood pressure and respiratory function) we like to refer to our original publications (Knippels et al., 1998a,b; 1999a,b; 2000).

In short for the sensitization studies young male Brown Norway (BN) rats were used, 4-6 weeks old at study initiation. As hen's egg white (HEW) proteins, as well as purified ovalbumin (OVA) from HEW, and cow's milk proteins (CM) were used for sensitization studies, the rats were bred and raised on a commercially available HEW-protein and CM-protein free rodent diet. To ensure the use of immunologically naive animals with respect to the antigens under investigation pre-study blood samples were always tested for HEW-protein and CM-protein specific antibodies.

For the oral sensitization of the animals they were exposed by gavage dosing to OVA, HEW-proteins, or CM-proteins (1 mg or 10 mg protein/ml tapwater; 1 ml/animal) during 6 weeks, without the use of an adjuvant. At weekly intervals blood samples were obtained from the orbital plexus or by exsanguination from the abdominal aorta at sacrifice. The sera were analysed for anti-OVA, HEW-protein, and CM-protein specific IgG titers by Enzyme Linked Immunosorbent

Assay (ELISA) and anti-OVA, HEW-protein, and CM-protein specific IgE by ELISA and passive cutaneous anaphylaxis (PCA)-test.

RESULTS

Immunological parameters

Daily intra-gastric administration of 1 mg OVA during 42 days, without the use of adjuvants, resulted in antigen-specific IgG as well as IgE responses in almost all rats. In general the percentage of IgE responders in our studies exceeded 80%, as meaured by ELISA and PCA. Optimal specific IgE antibody responses, were observed around day 28-35. Occasionally, however, no OVA-specific IgE responses were induced upon daily gavage dosing with OVA in our studies. Using less frequent administration regimes of 1 mg OVA by gavage in general did not induce specific IgG or specific IgE antibody responses. Upon ad libitum exposure, only OVA-specific IgG but no OVA-specific IgE was produced (Knippels et al., 1998a)

Although antigen-specific IgG responses were found upon daily gavage dosing of the animals to different concentrations of HEW or CM, only a limited number of IgE responders were observed as measured by PCA. However, immunoblotting experiments with these rat sera demonstrated specific-IgE antibodies against both HEW-proteins and CM-proteins (Knippels et al., 2000). Moreover, antibodies present in sera of orally sensitized rats to HEW or CM and in sera of allergic patients to HEW or CM recognized a comparable profile of allergens in these allergic food products. Specific IgG antibodies in sera from both hen's egg-allergic patients and rats orally exposed to HEW-proteins recognized a whole scale of proteins, although the profile appears to be the same. Despite the fact that egg white is a complex mixture of more than 20 proteins, the specific IgE antibodies in sera from both hen's egg-allergic patients and rats orally exposed to HEW-proteins recognized the same proteins, viz. ovotransferrin, ovalbumin, ovomucoid, and to a lesser extent lysozyme, which are claimed to be the major allergens for hen's egg allergic humans (Ebbehoy et al., 1995). No reaction was observed against any other protein present in the HEW-protein extract. The same phenomenon was observed when the pattern of protein recognition by antibodies in sera from rats orally sensitized to CM-proteins and antibodies present in sera from milk allergic patients was compared. Although cow's milk contains more than 30 proteins, the induced antibodies were mainly directed against β-lactoglobulin and, to a lesser extent, against the caseins, whereas no reaction was observed against any other protein present in CM. These studies indicate that upon daily intra-gastric dosing with HEW-proteins or CM-proteins, the specific protein recognition of induced antibodies in the BN rat is comparable to that observed in sera from allergic patients. The same phenomenon was described in BN rats intraperitoneally sensitized with CM which produced a profile of IgE antibodies to milk proteins similar to that observed in humans (Atkinson et al., 1996). Although, the induced antibodies in the BN rat apparently react to relevant proteins compared to the human situation, it remains to be elucidated whether the induced specific antibodies in the rat react to the same epitopes as the antibodies in the sera from patients. Furthermore, it should be recognized that the observed differences in responses to the different food proteins as observed for the experimental animals as well as for patients will probably be due to a combination of factors such as the dose of allergen, known to influence the outcome of oral sensitization, and the allergenicity of the proteins used (Atkinson et al., 1996; Pauwels et al., 1979; Strobel et al., 1984; Turner et al., 1990). However, the same allergens were recognized when compared to food allergic patients and

no antibodies were produced to the majority of other proteins present in CM and HEW-protein extract.

These results indicate that enterally exposed BN rats and young patients demonstrate IgE antibody responses to a comparable selection of proteins upon exposure to different protein mixtures and further support that the BN rat may provide a suitable animal model for assessing the allergenicity of (novel) food proteins and for research on mechanisms of food allergy. For a more detailed characterization of the developed rat model, additional studies were performed in OVA sensitized rats to study local and systemic immune-mediated effects upon an oral challenge with OVA (Knippels et al., 2000).

Immune-mediated effects in sensitized rats

Upon renewed oral antigen exposure of food allergic patients many different clinical signs or physiological reactions may occur ranging from oral allergy syndrome, effects on the gastro-intestinal tract physiology or on the respiratory and/or cardiovascular system to skin effects (Bruijnzeel-Koomen et al., 1995). Also in the BN rat model oral challenge reactions should be studied to show if, like in allergic patients, clinical reactions can be induced and involve like in patients various organs. Therefore, the animals previously sensitized with OVA were orally challenged 10 days after the oral sensitization period by intra-gastric intubation with doses of OVA varying between 10 and 100 mg. Subsequently effects on gastro-intestinal permeability, blood pressure, and breathing frequency were investigated (Knippels et al., 1999b).

The possible occurrence of local effects upon an oral challenge were studied by measuring gut permeability. Upon an oral challenge with OVA, gut permeability was increased as evidenced by an increased uptake of a bystander protein (β-LG). One hour after an OVA challenge followed by a dose of β-LG 30 minutes later, the amount of β-LG in the sera of previously sensitized rats was significantly higher compared to non-sensitized animals. Several models of intestinal hypersensitivity to food proteins have shown that antigen challenge of the sensitized intestine causes alterations in ion transport, permeability, and motility (Crowe et al., 1992; Berin et al., 1997). Also the release of mediators in anaphylactic reactions such as histamine, platelet-activating factor, prostaglandins, leukotrienes and some newly formed cytokines have been shown to alter mucosal function in experimental models (Heyman et al., 1994; Kanwar et al., 1994). Whether the increased macromolecular passage is mainly due to transcellular or paracellular transport is still not clear. However, Scudamore et al.(1995) showed that the release of rat mast cell protease-II, a known rat mucosal mast cell mediator, increases epithelial permeability via a paracellular route. Together with our finding that a significant amount of intact β-LG is present in sera of sensitized animals, this may suggests that the epithelial permeability is increased in our animals via the paracellular route, although an increased permeability via the transcellular route can not be excluded.

In addition to studies on local effects, the possible occurrence of systemic effects were investigated by monitoring respiratory functions and blood pressure. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of animals. However, immediately after challenge some animals demonstrated a temporary drop in breathing frequency that returned to normal rates within 10 min. In respiratory allergy studies in guinea pigs, a drop in breathing frequency below 70% of the normal breathing frequency is referred to as an indication of severe respiratory effects (Botham et al., 1989). Although, we only observed severe respiratory effects in a few animals (around 10-15% of the animals), this low incidence is in agreement with observations from food allergic patients, of whom only about 10% is reported to react with respiratory problems (Monteleone et al., 1997). Also the effects on

systolic blood pressure, which occurred in approximately 40 % of the challenged rats, were not dramatic and did not result in a circulatory collapse. Two different patterns of blood pressure drop could be distinguished, in which some animals showed a continuous decrease and other animals a delayed decrease. Again, the rather low incidence of cardio-vascular effects upon oral challenge of the rats is in accordance with the human clinical practice. These observations indicate that in orally sensitized animals systemic effects can be induced upon an oral challenge.

CONCLUSION

It is evident that new approaches are needed to more appropriately assess the potential oral allergenicity of "novel proteins". The development of a predictive animal model is in this context often indicated to be of major importance to improve the currently used ILSI/IFBC decision tree. In particular if transferred genes, coding for new proteins, are derived from products with an unknown history of allergenicity or if proteins show one or more physico/chemical characteristics of known allergens, the ultimate proof for presence/absence of sensitizing activity of the novel proteins can be studied in a predictive animal model. In the present paper studies are summarized focussed on the development of an oral sensitization protocol, in which without the use of an adjuvant Brown Norway rats were sensitized to various allergenic food proteins (Knippels et al., 1998a,b; 1999a,b; 2000). The results clearly indicate that oral exposure of BN rats to food proteins may result in a significant IgE response to the proteins. In addition, when the BN rats were exposed to a mixture of proteins (HEW- and CM-protein extracts) specific antibody responses were only elicited towards several proteins whereas to the majority of proteins present in CM and HEW-extract no specific IgE response was produced. The profile of allergens recognized by the immune system of the BN rat appeared comparable to the profile of allergens recognized by allergic-humans as measured by immunoblot techniques (Knippels et al., 2000). After an oral ovalbumin (OVA) challenge in the OVA sensitized BN rats also effects were shown on respiratory, circulatory, and gastro-intestinal functions. The possible occurrence of systemic effects upon an oral challenge were investigated by monitoring respiratory functions and blood pressure. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. However, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure which is in accordance with the human clinical practice (Knippels et al., 1999b). Following the studies on systemic effects, the occurrence of local effects upon an oral challenge was demonstrated by an increased gut permeability as demonstrated by an increased uptake of the bystander protein β-lactoglobulin 1,5 hour after an oral challenge.

Although additional studies are needed to further validate the developed Brown Norway rat model, the results obtained up till now indicate that the BN rat might be a useful and predictive animal model to study the potential oral allergenicity of "novel" food proteins. Moreover, for studying more mechanistic aspects of food allergy, that might result in better possibilities with respect to prophylaxis and therapy, the BN rat model seems to be promising too.

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