DRAFT SCIENTIFIC OPINION

Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed[1]

EFSA Panel on Genetically Modified Organisms (GMO Panel)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

This Opinion follows a request from the European Food Safety Authority (EFSA) to the Panel on Genetically Modified Organisms (GMO Panel) to establish a Working Group on "The Assessment of allergenicity of genetically modified foods".

The weight-of-evidence approach which is done on a step-by-step and case-by-case basis is considered the most appropriate way of assessing the allergenicity of GM food and feed. This report discusses various aspects to increase the strength and accuracy of this approach, including the latest developments pertaining to clinical aspects of allergic reactions, structural aspects of GM food and feed and *in silico* approaches, as well as IgE binding studies and cell-based methods, profiling techniques and animal models. In this context, conclusions and recommendations to update and complement the EFSA Guidance Document of the GMO Panel as regards the allergenicity assessment of newly expressed protein(s) and whole GM foods and feed are provided. In summary, it is recommended that with regard to the search for sequence homology and structural similarities, local alignment method with a known allergen with a threshold of 35% sequence identity over a window of at least 80 amino acids is considered a minimal requirement. When IgE binding tests are considered necessary, e.g. when there is sequence homology and/or structure similarity with known allergens, the use of compounds to be analysed in the comparative compositional analysis of the GM plant and its non-GM counterpart.

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Proposals for the use of additional testing that may improve the weight-of-evidence approach and suggestions for further assessment of new promising methods that are as yet in an early phase of development are also addressed.

The background information, scientific evidence and rationale on which the conclusions and recommendations are based, can be found in the annexes.

Annex 1 on clinical aspects discusses the mechanisms, the prevalence and clinical pictures of food allergy, as well as the diagnostic procedures. Within the assessment of allergenicity of GMOs, attention is paid to the risk for populations with a particular or altered digestive physiology such as infants. In addition, the possible role of GMOs as adjuvants, i.e. substances that, when co-administered with a protein increases its immunogenicity and therefore might increase as well its allergenicity, is considered within the assessment of GMO allergenicity. Where possible, the use of surveillance programmes such as post-market monitoring and survey of occurrence of occupational allergic reactions through different routes of exposure is proposed.

Annex 2 examines the structure and biological properties of a newly expressed protein in an integrative approach for assessing its possible allergenicity. Current understanding of how the allergenic potential relates to protein structure, biological properties, post-translational modifications and plant processing and may be affected by food processing and interactions with the food matrix is presented. This is discussed within the context of the natural variability and taking into account that genetics, environmental factors and post-harvest conditions may affect the expression of allergens in plants. Therefore, the considerations developed in this Annex do also pertain to the assessment of allergenicity of the whole plant.

Annex 3 reviews the various bioinformatics methods available including algorithms for search of sequence identity with known allergens and assessment of the relevance of alignments observed, and allergen databases. A recommendation is made that these allergen databases are regularly updated and checked for accuracy (e.g. absence of irrelevant allergens and presence of minor true allergens with low frequence of sensitization). Criteria for inclusion of proteins into allergen databases and for sequence identity searches are discussed as to improve the accuracy, sensitivity and specificity of current bioinformatics approaches. Bioinformatics analyses are not appropriate for the assessment of the *de novo* sensitization potential of a newly expressed protein but provide useful information on the possible IgE-cross-reactivity with known allergens. Such information should be confirmed by other tests to conclude on the likelihood of allergenicity of the newly expressed protein(s).

Annex 4 describes *in vitro* methods that can be used for assessing the allergenicity of newly expressed protein(s). This includes a discussion of different IgE binding assays and criteria for serum selection, and an overview of experimental conditions for performing and interpreting the outcome of pepsin resistance tests. IgE-binding assays aim to test for possible IgE-cross reactivity of the newly expressed protein with known allergens. Serum screening is required if there is any indication of relationship or structure similarity with known allergens as evidenced in the previous step of the assessment. In view of the problems associated with the use of sera of human origin, the possibility of using well characterized antibodies raised in animals for a pre-screening may be envisaged. Stability towards *in vitro* digestibility can provide additional information about the possible allergenic potential of the newly expressed protein. The conditions that should be observed for performing those tests and correctly interpreting the outcome are discussed in relation with the clinical considerations presented in Annex 1. Cell-based assays that employ either cells isolated from human or animal tissues or propagated from immortal cell lines are also considered.

Annex 5 addresses analytical methods and profiling techniques for assessing the potential increase of the intrinsic allergenicity of the whole plant and derived products as an unintended effect of the genetic modification. When the recipient of the introduced gene is known to be allergenic, a potential quantitative and/or qualitative change in the allergenicity of the whole GM food should be tested by

comparing the allergen repertoire of the GM plant with that of the conventional counterpart taking into account the natural variability in the endogeneous allergen expression. Allergens in whole plants can be analysed based on their immunochemical and biological properties with gel-electrophoresis followed by immunoblotting. Alternative proteomic methods using, e.g. mass spectrometry for identifying and quantifying allergens are also reviewed. High throughput analytical techniques are a promising tool for non-targeted profiling of differences in protein expression in the GM plant compared with its non-GM counterpart. It is proposed that they should be thoroughly assessed for accuracy, sensitivity, specificity and feasibility before being routinely used for allergenicity assessment.

Annex 6 reviews and makes a critical appraisal of the numerous animal models (including transgenic animals) that are currently available to test the capacity of a newly expressed protein to elicit an allergic reaction in allergic consumers already sensitized to a cross-reactive allergen or its potential to *de novo* sensitize predisposed individuals. Advantages and pitfalls of the different models are analysed. It is noted that none of these models fully reproduce either the diversity and variability of the IgE response in heterogeneous populations of allergic humans or the conditions of sensitization that occur in the real life upon given conditions of exposure and environment. In addition, none of the current animal models has both enough sensitivity and specificity in order to guarantee the absence of false negative and false positive results. Animal models are, therefore, in general considered not validated and inconclusive for the assessment of the sensitizing potential of a novel protein. However, they can provide useful information on the different mechanisms underlying the induction and development of an allergic reaction when there are indications of a sensitizing or adjuvant potential of the newly expressed proteins.

KEY WORDS

Allergenicity, genetically modified organism, food, feed, safety, newly expressed protein, weight-of-evidence approach.

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ASSESSMENT OF ALLERGENICITY OF GM PLANTS AND MICROORGANISMS AND DERIVED FOOD AND FEED

1. INTRODUCTION

1.1 Food allergy

Food allergy is an adverse reaction to food and represents an important public health problem. Food allergy is different from toxic reactions and intolerance. Allergy is a pathological deviation of the immune response to a particular substance which affects only some individuals where a combined effect of variations in the environment and genetic predisposition has resulted in allergic sensitization. In allergic individuals, sometimes minute amounts of a food that is well tolerated by the vast majority of the population can cause serious symptoms and death. It is not the allergen per se, but the allergic person's abnormal reaction to the allergen that causes the adverse health effect. Food allergy can be caused by various immune mechanisms. However, IgE-mediated food allergy represents the main form of food allergy, that causes the most severe reactions and the only form causing life-threatening reactions. Up to now this IgE-mediated food allergy has been the focus in the risk assessment of allergenicity of GMOs and Novel foods (2003; EC, 1997; EFSA, 2006b). With regard to food intolerance, the mechanisms are not well understood but in many cases is a consequence of a defect in digestion or metabolism.

The prevention and management of food allergy is a responsibility of governmental regulatory bodies as well as the food industry and the food suppliers. The assessment of allergenicity of a food or food component, that is the capacity to induce an allergy, is the responsibility of scientific risk assessment bodies, and is a prerequisite of marketing of GM foods (Codex Alimentarius, 2003).

1.2 IgE-mediated food allergy

Importantly, food allergy consists of two separate phases: first *sensitization* where no symptoms occur while the capacity of the immune system to react increases dramatically, and later *elicitation (provocation)* with clinical manifestations. When ingested, the allergen(s) i.e. the sensitizing food or food component is to some extent degraded by digestive enzymes, absorbed by the gut mucosa (small amounts even by the oral mucosa), processed in specialized cells of the immune system and then presented to the reactive immune cells that produce an immune response. Sensitization can also occur if the food allergen comes into contact with skin or is inhaled (Lack et al., 2003). The mechanisms of IgE-mediated food allergy are described in Annex 1. In susceptible individuals, previous exposure to the incriminated food resulted in the synthesis of specific antibodies by lymphocytes, including antibodies of the immunoglobulin E (IgE) class. The specific IgE antibodies circulate in the body and bind to the surface receptors of blood basophils and tissue mast cells. Subsequent contact of the immune system with the same allergen, or with another molecule sharing common structures (so-called epitopes) with the parent sensitizing allergen, will cause more specific IgE to be produced and to bind to basophils and mast cells. This first phase of allergy is the *sensitization phase*, during which the immune system responds with specific IgE production to the allergen in question, a process that by itself does not cause any symptoms. Finally, when the density of IgE antibodies on the mast cell and basophil cell surface is sufficiently high, the *elicitation phase* can occur. If the allergen now comes into contact with the IgE-coated immune system cells, the allergen molecule (as intact protein or at least bivalent immunoreactive fragments) will bind and cross-link the specific IgE present on the cell membrane. This cross-linking of IgE on mast cell and basophil cell membranes triggers the early phase of the immediate type allergic reaction. Pharmacologically active mediators, such as histamine, prostaglandins, and leukotrienes are released, causing the onset of the various symptoms of allergy. Some hours later, a second phase of inflammation may develop ("late phase reaction"), caused by a cellular reaction in which eosinophil granulocytes and T lymphocytes play a dominant role. The symptoms of the food allergic reaction are not limited to the oral cavity and the gastrointestinal tract

but are also cutaneous and respiratory and even cardio-vascular. It is noteworthy that some individuals may get sensitized to an allergen i.e. produce detectable amounts of specific IgE antibodies to this allergens without developing any clinical symptoms of allergy upon exposure to this allergen.

Because of structural similarity between certain allergens, the same food or allergen is not always required for both steps of the allergic reaction. Specificity is limited to the small part of the allergen (the epitope) directly binding to IgE. Epitopes on different proteins, as a rule evolutionary related, may be identical or similar enough to bind to the same IgE molecules. When sensitization to one allergen causes the immune system to respond to another allergen because of shared epitopes (identical or with a high degree of similarity) between the allergens, this is called cross-reactivity.

It is important to note that there are two levels of cross-reactivity. Cross-reactivity may be limited to IgE binding without observed adverse effects (cross-sensitization), or it may also confer clinical reactivity (cross-allergenicity). Cross-reactivity on the level of IgE binding is much more common and widespread than clinical cross-reactivity. The *in vitro* demonstration of cross-reactivity between two allergens in terms of IgE binding is no proof of clinical cross-reactivity, and only means that one important pre-requisite for clinical reactivity is present.

Cross-reactivity complicates food allergy assessment, management and prevention. Cross-reactions exist between foods, between foods and pollen, and between foods and other substances like rubber latex, mite and insect allergens. As an example, frequent cross-reactions are observed between birch pollen and hazelnut, apple and more generally fruits of the *Rosaceae* family. Cross-reactions are also observed between pollen of *Compositae* (mugwort) and celery. Also important are foods that cross-react with latex, e.g. chestnut, walnut, kiwi, banana, and avocado. Depending on their different epitope preferences, different individuals differ in their cross-reactivity patterns.

1.3 Mandate

The GMO Panel was given the mandate to establish an *ad hoc* Working Group for a self mandate on "Assessment of allergenicity of GM foods":

- to review current strategies and test methods for the assessment of allergenic potential of GM plants/microorganisms as food or raw materials for food ingredients;
- to consider new scientific developments and methodology in the area of allergenicity testing and assessment;
- as appropriate, to propose new approaches for the assessment of allergenicity, including more accurate and sensitive tests as to increase the level of safety assurance of non-allergenicity of GM plants/foods and demonstrate that the genetic modification does not increase the allergy risk;
- to invite external experts to the Working Group recognized for their competence in specific scientific fields and/or for their experience on case studies;
- to organise workshops with the stakeholders;
- to prepare recommendations to be used for the scientific assessment of allergenicity of GM plants and their products as an update/complement of the EFSA Guidance document for the risk assessment of genetically modified plants and microorganisms and derived food and feed (EFSA, 2006a, 2006b).

1.4 Allergenicity assessment of GMO's

Allergenicity is not an intrinsic, fully predictable property of a given protein but is a biological activity requiring an interaction with the immune system in predisposed individuals. It, therefore, depends upon the genetic diversity and variability in environmental exposures in the individuals. Given this lack of complete predictability it is necessary to obtain, from several steps in the risk assessment

process, a cumulative body of evidence which minimises any uncertainty with regard to the protein(s) in question. In line with the recommendations of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology (Codex Alimentarius, 2003) and the EFSA guidance document (EFSA, 2006b), an integrated, stepwise, case-by-case approach, as described below, should be used in the assessment of possible allergenicity of newly expressed proteins.

These new proteins can either *de novo* sensitize individuals particularly those with a predisposing genetic background (i.e. atopic individuals) and history of environmental exposure and/or elicit an allergic reaction in individuals already sensitized to another cross-reacting protein. Increased exposure to allergens already present in the conventional crop may also be part of the risk profile of GM plants, as the genetic modification may have resulted in unintended changes in the pattern of expression of endogenous allergens (e.g. over-expression of endogenous allergenic proteins naturally present in the recipient plant).

Consequently, when assessing the allergenicity of GMOs, two main issues are clearly to be addressed (EFSA, 2006b): i) the allergenicity of the newly expressed protein(s) that can be present in edible parts of the plants (an issue related to the source of the transgene/newly expressed protein, and ii) the potential increase of the intrinsic allergenicity of the whole plant and derived products as an unintended effect of the genetic modification (an issue related to the recipient). Another issue to be taken into account is a possible increase in the intake of / exposure to the GM food although its content of allergenic proteins has not been changed. Similar issues pertain to exposure to pollens of GM plants.

The allergy risk assessment of GMOs is based on a comparative approach. It aims to establish whether the allergenicity of the GM plant is similar or different to that of the non-GM counterpart. Since no single experimental method yields decisive evidence for absence of allergenicity, a weight-of-evidence approach is recommended taking into account all of the information of different nature obtained with various test methods (Fig. 1). Methods used for the allergenicity assessment of newly expressed proteins in GMOs first investigate whether or not the source of the transgene/newly expressed protein has a history of allergenicity, then include a search for sequence homologies and/or structural similarities between the newly expressed protein and known allergens, *in vitro* tests to measure the capacity of specific IgE from serum of allergic individuals to bind the test protein and the resistance to degradation by the proteolytic enzyme pepsin. Where necessary, other additional tests may be used. These methods provide information on the risk of elicitation of an allergic reaction by the newly expressed protein in already sensitized individuals. Their contribution to the assessment of the risk of *de novo* sensitization of atopic individuals is still a matter of scientific debate.

As the pre-market assessment of GM products under Regulation (EC) 1829/2003 also considers the use of a GM product as animal feed besides food, the potential for allergenicity in animals, both companion and livestock, should be considered. Allergy and intolerance to feeds in animals may be due to several mechanisms and result in various clinical manifestations. The pathophysiology, the nature of the most common allergens, the level of exposure and digestive physiology in animals may be different from those in humans. Whilst animals and humans may share some allergens in common, no available source is known to exist to date that provides comprehensive information on specific compounds that would be allergic to some animals but not to humans.

1.5 Scope of the report

It is within the scope of this report to evaluate the robustness of currently used methods and to assess new scientific developments to refine the current allergenicity assessment approach, as well as the feasibility of incorporating these in the above mentioned weight-of-evidence approach. Emerging new tools, both *in vitro* and *in vivo*, potentially appropriate for a more accurate and sensitive assessment of allergenicity, but not yet validated in routine will be evaluated.

The emphasis of this report is on the assessment of allergenicity in humans, defined as IgE-mediated or immediate-type hypersensitivity reactions. However, non-IgE mediated adverse reactions, immunogenicity, adjuvanticity, celiac disease and other food induced enteropathies have also been touched upon. Whilst the report focuses on allergenicity in humans mostly, it also considers allergenicity in animals, where applicable.

The above mentioned considerations will be reviewed and evaluated for novel proteins expressed in GM plants and microorganisms, as well as for food and feed derived from such organisms. They pertain to plants genetically modified for improved agronomic performance (herbicide tolerance, insect resistance or a combination of these traits) or for improving human or animal nutrition and/or health, but also to GM micro-organisms. The assessment of whole foods and feed is however difficult since food or feed products are complex. They may contain natural compounds, e.g. proteins that may be bioactive or allergenic *per se*. They may interact with the food matrix which would alter their functionality. In addition, processing may affect their structure, digestibility and therefore their allergenicity in the processed foods. The assessment of the allergenicity of the whole food or feed will mainly focus on the comparison of the qualitative and quantitative patterns of expression of endogenous allergens in the GM plant and the non-GM counterpart.

2. STRATEGY FOR ASSESSING THE ALLERGENICITY OF GM FOOD AND FEED

This section summarizes the strategy recommended by the EFSA GMO Panel for assessing the allergenicity of GM foods and feed. It follows the same integrated, stepwise, case-by-case approach as recommended by Codex Alimentarius (2003) and as described in the EFSA guidance document (EFSA, 2006b). Following the analysis of the conclusions of its self mandate Working Group on "Assessment of allergenicity of GM foods", the EFSA GMO Panel is still of the opinion that the weight-of-evidence approach is the most appropriate way of assessing the allergenicity of GM food and feed. With the purpose of updating and further refining this approach, a review has been made of the latest developments regarding clinical characteristics of food allergy, structural aspects of GM food and feed, *in silico* (or bioinformatic) approaches, IgE binding and cell-based methods, analytical "profiling" techniques and animal models. Recommendations to update and complement the EFSA Guidance Document as regards the allergenicity assessment of GM food and feed have been provided. In addition, recommendations for further development related to methods in an early phase of development, or to methods that have not yet been fully explored and validated in the area of allergenicity assessment are also briefly addressed in this section.

So far, few tests have been validated for predicting the allergenicity of a (novel) protein or food. Validation may apply to the procedure in order to ensure the reproducibility of the tests and/or the relevance of the test. In this regard, the sensitivity of the test which reflects the rate of false negative results (*i.e.* the number of true allergens that would not be identified as such) and the specificity which reflects the rate of false positive results (*i.e.* the number of non-allergenic constituents that would be considered as allergens) are most important characteristics. No test with sufficient sensitivity, specificity and reproducibility is available to facilitate a definite conclusion on the allergenicity of a novel protein/food. However, the combination of a variety of tests may provide sufficient information on the likelihood of allergenicity for a risk assessment.

It is therefore emphasized that some of the recommendations provided, particularly in the additional testing sections are suggestions/proposals in order to add to the weight-of-evidence approach to demonstrate what risk of allergenicity there may be.

The recommendations are aimed, where applicable, at applicants, risk assessors, risk managers, policy makers and the scientific community. They are not, however, aimed at providing guidance to clinicians on protocols that should be performed in the diagnosis of an allergenic response of human consumers to a GM food.

The scientific evidence and background information, on which the conclusions and recommendations are based, as well as a more extensive description of the recommendations, can be found in the annexes as indicated in the footnotes and in the different sections mentioned below.

2.1 Assessment of allergenicity of newly expressed proteins

As indicated in the EFSA Guidance Document (EFSA, 2006b), it is essential that the tested protein is equivalent with respect to structure and activity to the newly expressed protein in the GM plant. Studies carried out using purified target proteins prepared by expression in organisms such as *Escherichia coli*, need to be related to the properties of the protein as expressed in the plant, thus taking into account all post-translational modifications that specifically occur in the plant.

In addition, adjuvanticity, which is the capacity of a substance that when co-administered with an antigen increases the immune response to that antigen, has not been routinely considered in the assessment of allergenicity of GMOs. It should be assessed when there are indications that the newly expressed proteins functionally or structurally resemble a known IgE adjuvant and are present at concentrations sufficient to be of concern.

2.1.1 Search for sequence and structural similarities[4]

Within the weight-of-evidence approach, a search for amino acid sequence and structural similarities between the newly expressed protein and known allergens shall be carried out using bioinformatics methods to identify IgE cross-reactivity between the newly expressed protein and known allergens. A typical *in silico* risk assessment of allergenicity minimally requires the following two resources: a repository of all known allergens with determined amino acid sequence (and, possibly, 3D structure) and an appropriate algorithm for searching relevant similarity between a query protein and the allergen database.

Criteria for inclusion of allergens into databases are rarely stated and therefore the quality of most databases is difficult to assess. Moreover, most of the existing databases are likely to contain errors, such as presence of irrelevant allergens, and absence of true allergens (e.g. that might be minor allergens in a food). Other important features of databases include good technical maintenance, regular curation, as well as a user-friendly retrieval system so that amino acid sequences can be easily extracted from the databases.

The alignment-based criterion involving 35% sequence identity to a known allergen using a sliding window of 80 amino acids as proposed by FAO/WHO, is still accepted as reasonably adequate for allergenicity/IgE-cross reactivity assessment also when compared to novel approaches. In addition, to optimize sensitivity versus specificity, a decision has to be made on the acceptability of false-negative rate since an overly strict adherence to sensitivity will result in an unreasonable number of false positives without completely avoiding all cross-reactivity risk. Peptide match of complete identity over 6 contiguous amino acids to known allergens is associated with very poor specificity (many false positives) and its relevance is doubtful. Complementary methods could also be considered to further ensure absence of similarity to known allergens. For example, several web servers that rely on novel principles (based on motifs and peptides specific to allergens) have shown to be highly specific without loosing in sensitivity. In addition it is noted that the production of IgE antibodies depends on activation of specific T lymphocytes. A search for homology between T cell epitopes with known allergens could therefore be carried out.

Conclusion:

[4] See annex 3

1. There is an important development in bioinformatics methods that are widely used for the risk assessment of newly expressed proteins in GMOs.
2. The information provided by *in silico* tests is in principle considered useful and reliable for the search of amino acid sequence identity between newly expressed proteins and known allergens. However, many different bioinformatics methods are currently in use.
3. Methods searching a complete identity over 6 contiguous amino acids to known allergens are associated with very poor specificity (many false positives). Therefore, it is in general not considered appropriate.

Recommendation:

1. Allergen databases used for the assessment of the risk of cross reactivity should be as comprehensive and quality assured as possible (see Annex 3.4; 3.12). An international agreement on regularly updating the databases should be considered.
2. There is a need for standarisation and harmonisation in search strategy and interpretation. The alignment-based criterion involving 35 % sequence identity to a known allergen over a window of at least 80 amino acids is considered a minimal requirement for risk assessment and is strongly recommended, although the identity threshold is conservatively set. Calculation of percent identity should be performed so that inserted gaps are treated as mismatches (see Annex 3.7.1; 3.12).
3. In addition, with regards to clinical considerations as detailed in Annex 1, it is noted that there is a need to consider data regarding molecular structures of not only proteins known to be responsible for IgE mediated allergy but also for other types of immuno-mediated or immuno-toxic reactions such as peptide fragments of gliadin involved in celiac disease (see Annex 1.10.2).
4. It is also proposed that the human allergen databases used for collecting allergen sequences for bioinformatics-supported comparisons of newly expressed proteins to allergens is extended with allergens for animals (see Annex 1.10.4).

Recommendation for further development:

Future work should focus on the completion of databases with information on 3-dimensional conformation of the allergens.

2.1.2 IgE binding tests[5]

The following step for assessing the potential that exposure to newly expressed proteins in GMOs might elicit an allergic reaction in allergic individuals already sensitized to cross reactive proteins, is based on *in vitro* tests that measure the capacity of specific IgE from serum of allergic individuals to bind the test protein(s). It is noteworthy that an IgE binding capacity which is identified for a newly expressed protein may not always imply that this protein would trigger an allergic reaction with clinical manifestations.

If the source of the introduced gene is considered allergenic but no sequence homology of the newly expressed protein to a known allergen is demonstrated or if the source is not known to be allergenic but there is any indication of relationship between the newly expressed protein and a known allergen, based on sequence homology or structure similarity, specific serum screening should be undertaken with sera from individuals with a proven allergy to the source or to the potentially cross reacting allergen using relevant immunochemical tests. IgE-binding assays (such as Radio or Enzyme Allergosorbent Assay (RAST or EAST), Enzyme Linked Immunosorbent Assay (ELISA) and electrophoresis followed by immunoblotting with specific IgE-containing sera) are adequate methods. It is noted that there is inter-individual variability in the specificity and affinity of the IgE response. In particular the specificity of the IgE antibodies to the different allergens present in a given food/source

[5] See Annex 4.3

and/or to the different epitopes present on a given protein may vary amongst allergic individuals. Also sera from clinically well characterized allergic individuals that are the reference material for IgE binding studies may be available in limited number and quantity. It is noted that phage libraries of human antibodies are currently available and could be used to create a GMO-specific library of human antibodies for pre-screening. In order not to test only the IgE binding capacity of the newly expressed protein but also its functionality to act as an allergen, the basophil degranulation assay is highly sensitive and specific. This test can be standardized using cell lines transfected with the human FcεRI receptor, such as basophil rat leukemia cells.

Conclusion:

1. Relevant human sera may be difficult to obtain and are often limited in number and quantity. However the use of human sera is necessary, in some specific circumstances, and on a case-by-case basis. When it is to be used the following is recommended.

Recommendation:

1. Human serum screening tests should be carried out with sera from well characterized individual patients in order to reflect the wide pattern of specificity of the IgE response and to evidence potential IgE binding to minor allergens by sera from a few individuals, which would be masked if the test is performed on pooled sera because of their dilution (see Annex 4.3; 4.6).

Recommendation for further development:

Future work should focus on the developement of cell based tests for assessing the capacity of the newly expressed protein to bind IgE and provoke the degranulation of basophils.

2.1.3 Additional testing

As indicated in the EFSA GMO Guidance Document (EFSA, 2006b), if no IgE binding is observed, additional testing such as a pepsin resistance test should be performed. This document provides some guidance on how these tests could be carried out as to provide more accurate information.

2.1.3.1 Pepsin resistance test[6]

Many food allergens that sensitize through the oral route display certain stability during digestion. Although a correlation between the resistance of a protein to proteolytic degradation by pepsin and allergenic potential has been reported, digestibility cannot be completely predictive. Protein stability in *in vitro* digestibility tests should therefore still be considered a risk factor but not in isolation, i.e. the outcomes of the digestibility assays should be interpreted in conjunction with results of other assays and other properties of the protein under consideration. In addition, the outcomes of the *in vitro* digestibility tests should be interpreted with care as they represent model conditions. *In vitro* procedures usually do not reflect the fluctuations in pH values and enzyme to protein ratios that occur *in vivo* after consumption of a meal. Interpreting the outcomes of the *in vitro* digestibility studies on the isolated newly expressed protein in the light of other factors, such as the abundance of the protein within the food, interactions with the food matrix and possible structural/functional alterations occuring during food processing would certainly be useful.

The specific risk of allergenicity of GM foods in infants as well as in individuals with impaired digestive functions should be considered and therefore, the differences in the digestive physiology in these subpopulations should be taken into account. Primary sensitization in the gut of young infants might be favoured by the immaturity of the local immunity and incomplete barrier function of the intestinal gut mucosa as well as incomplete protein degradation by pepsin in the stomach due to a gastric pH above values seen in adults.

[6] See Annex 4.2

Conclusion:

1. Several *in vitro* digestibility tests are available, and resistance of protein to digestion is still considered as informative with regards to allergenicity of a test protein.
2. As it is currently performed, a pepsin resistance test is not fully predictive for the allergenicity of the newly expressed protein.

Recommendation:

1. The pepsin resistance test or other *in vitro* digestibility tests should be performed in more physiological conditions in order to take into account variations of the pH value, the enzyme:protein ratio in the stomach and the impact of the food matrix and processing on the digestibility of the protein (see Annex 4.2; 4.5).
2. The *in vitro* methods for measuring the resistance of newly expressed proteins to pepsinolysis should take into account the conditions of individuals with modified digestion such as pH values > 2. Besides the oral route of sensitization, also other possible routes of sensitization (e.g. via respiratory or cutaneous exposure) may be considered (see Annex 1.8; 1.10.1).
3. The occurrence of stable protein fragments and/or of potential larger fragments containing re-associated peptides during digestibility testing should be considered as a risk factor and should therefore be looked for. In the case that detection methods, such as gel electrophoresis, are insufficient to detect such new compounds, the use of alternative methods (e.g. HPLC and MS) is recommended (see Annex 4.5.1).

2.1.3.2 Targeted serum screening

Targeted serum screening pertains to the use of sera in which IgE are present, directed towards allergens that are broadly related to the source of the transgenic protein. This had been recommended by the FAO/WHO consultation in 2001 but guidance on the procedures and interpretation of the outcome are still not well defined and the test has not been commonly used so far.

2.1.3.3 Animal models[7]

Animal models may be useful for the assessment of the allergenicity of newly expressed proteins. Whereas some models are more focused towards the study of the *de novo* sensitization by a novel protein, other models are rather designed to study the potential capacity of a protein to elicit an allergic reaction in animals previously sensitized to a cross reacting protein or whole food. It is noteworthy that none of these models fully reproduce either the diversity and variability of the IgE response in heterogeneous populations of allergic humans or the conditions of sensitization that occur in real life upon given conditions of exposure and environment. In addition, no animal model has both high sensitivity and specificity in order to guarantee the complete absence of false negative and false positive results. Therefore, animal models are often considered not validated and inconclusive for the assessment of allergenicity. Indeed no single model can provide definite conclusion on the allergenicity of a novel protein or a GMO, neither in terms of likelihood/frequency nor in terms of severity. In case there is indication from the origin or from the structure of the newly expressed protein that it might act as or like a sensitizer, the potential of the newly expressed protein to sensitize *de novo* atopic individuals could be assessed only using animal models. Animal models might also be useful to study the cross-reactivity of the newly expressed protein with known allergens and the clinical relevance of observed immunological cross-reactions, e.g. by investigating the potential of the newly expressed protein to elicit an allergic reaction in animals previously sensitized to a cross-reacting allergen. All the different animal models available (e.g. using different species and/or different procedures of sensitization) have advantages and pitfalls and therefore are not conclusive per se. However they can provide useful information on the different mechanisms underlying the induction and development of an allergic reaction. In the future, two types of animal models are likely to become of most interest in the search for GMO allergenicity : humanized mouse models in which

[7] See Annex 6

major histocompatibility class II complexes are entirely of human origin and mouse strains which have been made deficient in innate immunity components. They will provide information likely to be extrapolable to the human situation.

Same considerations apply for the assessment of adjuvanticity of the newly expressed protein when indications exist for such a potential. Although there is no definite test for adjuvanticity and variability between species are observed, animal models may provide preliminary information.

Conclusion:

1. Many animal models (including transgenic animal models) have been and are currently developed for sensitization, elicitation and adjuvanticity testing using different species and procedures. However, none of them fully reproduce either the diversity and variability of the IgE response in heterogeneous populations of allergic humans, or the conditions of sensitization that occur in the real life upon given conditions of exposure and environment. Animal models are therefore frequently considered not validated and inconclusive for the assessment of allergenicity.
2. Nonetheless, animal models can provide useful information on the different mechanisms underlying the induction and development of an allergic reaction.

Recommendation:

1. Antibodies obtained from animals experimentally sensitized in well defined conditions could be used as a substitute for allergic human sera for a (pre)screening of the immunological cross-reactivity of the newly expressed protein with known allergens (see Annex 6.4).
2. In specific cases such as when indications for sensitization or adjuvant potential exist, additional information gained from (combination of) animal models might be useful (see Annex 6.4).

Recommendation for further development:

Future work should aim to improve the sensitivity and specificity of tests on animal models as to allow consistent and reliable conclusion on a sensitization and/or adjuvant potential and explore the use of transgenic animals which are likely to develop *de novo* sensitization to newly expressed proteins and are extrapolable to the human situation.

2.2 Assessment of allergenicity of the whole GM food

As stated in the EFSA Guidance Document, when the recipient of the introduced gene is known to be allergenic, any potential change in the allergenicity of the whole GM food should be tested by comparison of the allergen repertoire of the GMO with that of the conventional counterpart. The assessment of the allergenicity of the whole GM food does not directly refer to the weight-of-evidence approach as described above and it should be conducted in addition to the assessment of allergenicity of the newly expressed protein. The allergy risk of GMOs may be associated to unintended effects that may impact on the overall allergenicity of the plant, e.g. resulting in an over-expression of natural endogenous allergens.

This implies that for allergenic plants or derived foods, the comparative compositional analysis of the GM plant with that of the non-GM counterpart should include known allergens, using targeted or possibly non-targeted profiling techniques.

2.2.1 Quantification and characterization of allergens within the compositional analysis

In principle, the allergen repertoire of the GM plant and of its non-GM counterpart should be analysed and compared, taking into account the natural variation in the levels of expression of allergens in edible tissues (and pollen) of the plant and the possible presence of isoforms. Although the number of identified food allergens has increased tremendously in the recent past, little is known about actual

allergen concentrations of allergens for individual foods. Because of the great natural variability in the expression of the endogenous allergens, differences between non-GM cultivars may be higher than differences between the GM plant and its non-GM counterpart. Including allergens in the list of compounds to be determined in the comparative compositional analysis would provide useful information. In order to assess the possible direct or indirect impact of the genetic modification, data on the level of expression of the endogenous natural allergens (as well as of the newly expressed proteins) in different tissues need to be established. In addition, indications on how agronomic conditions, developmental stage and post-harvest storage alter expression levels in the GM and non-GM plant and how the food matrix and technological processing may affect the allergenicity of the whole GM and non-GM food need to be further investigated (see Annex 2). Establishing the qualitative and quantitative allergen profiles would require a set of appropriate analytical methods that may employ either targeted or non-targeted approaches for the analysis of whole protein extracts prepared from the GM plant and its non-GM counterpart(s).

Allergens can be analysed based on their immunochemical properties by different formats of direct and/or competitive inhibition immuno-assays such as ELISA and/or two dimensional electrophoresis in gel (2DE) followed by immunoblotting using sera of individuals allergic to the recipient plant. However, the sensitivity of such tests may be too low to identify alterations in the expression of particular allergens in the GM versus the non-GM plant (see Annex 4).

High throughput technologies including novel mass spectrometry-based methods are a potent tool for alternative non-targeted proteomics studies which may allow comparing the whole proteome of GM and non-GM plants. However, even if promising, available data are still preliminary and the techniques in question need to be further investigated and validated before being routinely used in the allergenicity assessment process (see Annex 5).

Conclusion:

1. Qualitative and quantitative patterns of expression of endogenous allergens need to be established in common non-GM cultivars in order to perform a comparative compositional analysis with GM plants.
2. Relevant and adequate analytical tools are available to quantify and characterize the allergens expressed in the GM *versus* the non-GM plant, for which IgE binding tests are required.
3. High throughput analytical proteomic techniques are under development and may become useful for non-targeted approaches.

Recommendation:

1. It is recommended that allergens are included in the the list of compounds to be determined in the comparative compositional analysis of the GM plant and its non-GM comparator. Despite that great natural variability may occur, this should not preclude identification of any consistent unintended effect due to the genetic modification. Data on the level of expression of these allergens in edible tissues and pollen, and indications of how agronomic conditions, developmental stage and post-harvest storage affect expression levels and structure need to be established (see Annex 4.8).
2. To improve the available analytical tools for a comprehensive comparison of the allergenicity of the GM and non-GM plant, it is recommended to test appropriate protein extraction procedures in order to cover the different allergenic proteins present in the GMO and its non-GM counterpart, including those not easily soluble in aqueous buffers (see Annex 5.6.1; 5.7). With regard to IgE binding studies, a pre-screening might also be performed with the whole plant (protein extracts) using animal sera instead of human sera (see Annex 6.4).

Recommendation for further development:

A comprehensive study of the specificity, sensitivity and feasibility of non-targeted profiling technologies based on so called “omics”-techniques for the assessment of allergenicity needs to be performed and a thorough assessment to be made of their advantages and weaknesses in

order to compare these modern techniques with those of classical targeted analytical techniques in terms of reliability of interpretation of the results.

2.3 Other considerations

Recent progress in our understanding of the pathogenesis of food allergy and related diseases has confirmed the key role of IgE antibodies, but also of regulatory mechanisms involved in natural and acquired tolerance to foods. It is generally recognized that the prevalence of food allergy is increasing, and the clinical pictures are changing. Accordingly, improved diagnostic procedures must be developed and also involve non-IgE-mediated reactions. They should be related to the clinical reactivitiy i.e. the outcome of standardized food challenges in selected allergic individuals. The clinical assessment of food allergies in relation to GM foods should rely on observations provided by doctors directly in charge with food allergic individuals. To obtain a high level of vigilance, proper continuous education and clear information on the GM foods available on the market need to be provided to the health professionals and allergic consumers. Inclusion of GM foods in prevalence studies designed in a general context of protection of public health and survey of allergic diseases would also provide useful information.

Post-market monitoring programmes (PMM) aim to provide reliable information regarding actual intake of a novel food by different groups of consumers and occurrence of any expected or unexpected (adverse) effects in every day life conditions of consumption. It is recognized that there is a need to clearly inform medical doctors and consumers of the presence of GM- and non-GM- products but in line with the EFSA Guidance Document, the implementation of PMM can be recommended only on a case-by-case basis. In addition, information should be recorded and provided on any case of occupational allergic reaction that would be observed in individuals in contact with the newly expressed proteins or the GMO through different routes of exposure since routes and levels of exposure may affect the sensitization potential. Although the number of identified food allergens has increased tremendously in the recent past, little is known about the potency of individual allergens with regards to threshold levels of allergens set for sensitization and elicitation of allergic reactions by individual foods.

Conclusion:

1. Little information is available with regard to the actual exposure and threshold or sensitization or elicitation in different segments of the population consuming GM foods.

Recommendation:

1. Clear information needs to be provided to the consumers and health professionals on the GM foods available on the market.
2. PMM should only be recommended on a case-by case basis, e.g. for GM food/feed with altered nutritional composition and modified nutritional value and/or modified to achieve specific health benefits (see Annex 1.9.3; 1.10.6).

Recommendation for further development:

It is recommended that in case PMM is needed, (a) protocol(s) is/are developed to provide data on the actual intake of the GM food, to guarantee the relevance of the reported adverse effects and to allow establishing any relation/causalities with the consumption of GM foods. It could for instance be valuable to develop prevalence studies in which GM foods are included as potential allergens in order to have a quantitative estimation of the potential impact of cultivation and consumption of GM crops on the prevalence of allergies.

Finally, a more general recommendation which does not only apply for GMOs but for all food allergens is that further research is needed to determine thresholds for sensitization in man and thresholds for elicitation particularly in the case of cross-reactive allergies. This would allow identifying whether there is a level of expression of an allergen in a (GM) food that could be considered “safe”.

2.4 General conclusion

The strategy summarized in this report for assessing the allergenicity of GM foods and feed considers the allergenicity of the newly expressed proteins, the whole GM food, and also some other considerations, such as the exposure and intake of GM foods. Particularly with regard to newly expressed proteins, it is based on an integrated, stepwise, case-by-case approach, in line with the approach followed in the EFSA guidance document and the Codex guidelines. The different steps for the allergenicity risk assessment of GMOs are discussed in this report, and where relevant, additional recommendations are provided to further strengthen the weight-of-evidence approach. These include recommendations with regard to the search for sequence homology and structural similarities, IgE binding tests, the testing of the digestibility of newly expressed proteins, and the comparative analysis and structural aspects of endogenous allergens in whole GM foods. They are summarized as follows:

- To optimize the search for sequence homology and structural similarities, it is recommended to have allergen databases that are as comprehensive and quality assured as possible. In general, the alignment based criterion involving 35% sequence identity to a known allergen over a window of at least 80 amino acids is considered a minimal requirement.

- When *in vitro* tests that measure the capacity of the newly expressed protein to bind serum IgE from allergic individuals are considered necessary, it is recommended to use individual sera from clinically well characterized individuals rather than pooled sera.

- *In vitro* digestibility tests should be performed in more physiological conditions than is currently carried out in order to take into account not only variation of the pH value and the enzyme-to-protein ratio in the stomach but also the impact of the food matrix and processing on the digestibility of the protein. In addition, the digestion conditions in infants and in individuals with altered digestive physiology should be considered.

- With regard to the assessment of the allergenicity of the whole GM food, it is recommended that allergens are included in the the list of compounds to be determined in the comparative compositional analysis of the GM plant and of its non-GM comparator, and to perform a qualitative and a quantitative comparision of the allergen repertoire of the GM and the non-GM counterpart taking into account the natural variability in common cultivars.

In addition, suggestions have been made with regard to other additional testing, e.g. animal models, and with regard to further developments, particularly on 3-dimensional structure of allergens, profiling technology, post-market monitoring and exposure assessment.

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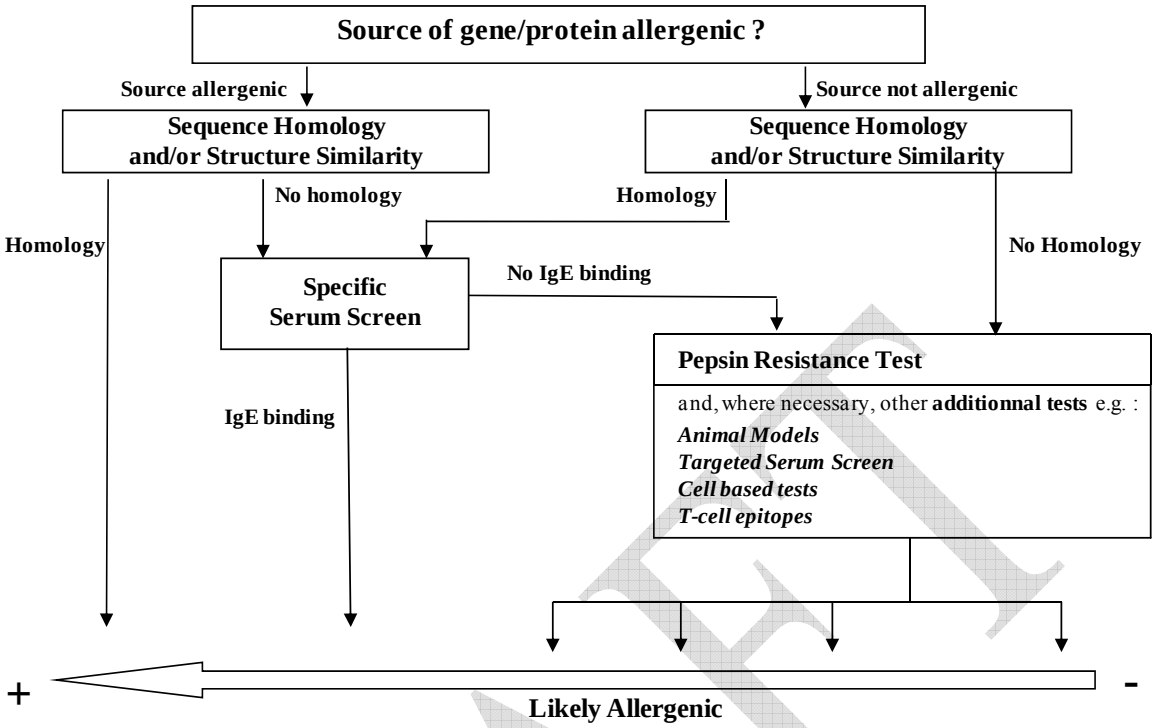

Figure 1: Flow chart summarizing the weight-of-evidence approach for assessment of allergenicity of newly expressed proteins in GMOs (Codex alimentarius, 2003; EFSA, 2006

ANNEX 1. CLINICAL ASPECTS OF FOOD ALLERGY

1.1. Introduction

"Food allergy" is often used to encompass many problems which do not involve allergic mechanisms. It is therefore important to put the problem into context. An adverse reaction to foods may occur predictably in all the population to a greater or lesser extent when the food contains bioactive substances such as histamine which can accumulate in scromboid fish such as tuna and will produce a reaction identical to that seen during the course of an allergic reaction. However, this will only occur if the tuna has been badly stored and will affect all people that ingest it. Other foods contain pharmacologically active ingredients such as caffeine in coffee, theobromines in chocolate and of course alcohol. Again the responses to ingestion of large doses are very predictable. Other foods can become contaminated with toxins generated by bacteria such as that associated with *Staphylococcus* which will produce vomiting and abdominal pain within an hour of ingestion. These predictable reactions must be discriminated from those which only occur in some people but not others when exposed to the same food. The most common reaction is psychologically based and might be described as food aversion. Thus known exposure to the food results in a range of bodily reactions including nausea, vomiting, abdominal pain, diarrhoea, respiratory symptoms. However, the reaction is not reproducible when the food is presented in a concealed form. If the reaction is reproducible then it can be termed as food intolerance where the mechanism is unknown or where it is associated with some form of error of digestion or metabolism. This is best exemplified by lactose intolerance where the digestive enzyme for the milk sugar, lactase is missing from the bowel, particularly in adults. This leads to the generation of lactic acid by bowel bacteria which in turn is intensely irritant to the bowel and produces abdominal pain, bloating and diarrhoea. Finally, the term food allergy may be applied to a reproducible adverse reaction to food where an immune mechanism can be implicated. While this is generally assumed to be associated with the production of IgE antibodies to the relevant food other immune mechanisms may be involved. This has led allergists to define two sub-groups of food allergy as IgE-mediated and non-IgE-mediated. Allergy itself which is synonymous with hypersensitivity may be defined as an altered acquired specific capacity to react. This implies that the immune system has modified its response following a primary exposure leading to an accentuated sequence of immune events which clearly is important in conferring immunity against repeated exposure to infectious agents but if the response is directed against innocuous factors such as foods then it can lead to the generation of a wide range of symptoms.

The immune system is highly sophisticated and immensely complex. It has evolved to handle attacks by damaging factors such as bacteria, viruses, fungi etc. The system achieves this by recognising danger signals. These are generated by two mechanisms, the first being as a consequence of the initial damage caused when the microorganism first enters the body. The damaged cells release chemical messages which attract the immune cells to the site of the attack and activate them so that they begin to initiate an immune response which will eventually eliminate the organisms. Secondly the immune active cells are able to recognise specific molecular patterns expressed by the infecting organisms. This is with receptors known as pattern recognition molecules. These are present both in the circulation, on the surface of immune active cells and even inside cells. The molecular patterns they recognise include infecting organism DNA sequences, molecules expressed in the cell wall of bacteria and many others. Once the molecular pattern is recognised a sequence of events occurs which results in the immune system not only generating chemicals known as cytokines which will eliminate the organism by innate mechanisms but also initiates a so-called adaptive response. This is the response where the immune system is now able to immediately recognise the invading organism and to rapidly mobilise the cells which will eliminate it before it is able to do any damage. It is this component of the response that can sometimes be subverted to react to harmless factors and produce inappropriate inflammation and disease. Thus the hypersensitive response to an infection is no different to the hypersensitive response that might be associated with allergy.

1.2. Food allergens

The term "food allergen" refers both to the complex whole food and to the chemically defined compounds that are responsible for allergenicity, i.e. the proteins. In the regulatory context, one usually deals with foods or classes of foods (e.g. "fish and products thereof"). However, the biochemical molecular entity causing most food allergies is proteins. Some protein breakdown products, *i.e.* peptide fragments, may conserve part of the allergenicity of the native protein and thus can also be considered as allergens. Food allergens are generally proteins of molecular weight of more than 9 kDa (but sometimes peptides may cause a reaction). As soon as a protein is consumed, there is a potential risk of allergy in particular in a genetically predisposed (atopic) consumer. Allergenicity of a given complex food is very rarely due to a single protein component but rather to numerous different proteins which constitute the "allergen repertoire" of the food. Due to the diversity and variability of the human IgE response, all of the allergenic proteins are not always recognised by all individuals allergic to this food. Those allergens that are recognised by more than 50% of a population of individuals allergic to the food are called *major allergens*. This concept relates only to the frequency of recognition by IgE antibodies, and it is not related to the severity of the clinical manifestations of an allergic reaction. Major allergens may constitute a small proportion of the total protein content of the food concerned. Clinical reactions may be similar whether they are triggered by major or minor allergens.

The allergenicity of each single protein is due to a number of molecular immunoreactive structures, the IgE-binding epitopes already mentioned. These epitopes comprise a limited group of amino acids.

Most allergenic epitopes consist of amino acids as added during transcription/translation of DNA to RNA to protein (translational peptidic epitopes). However, epitopes may also be formed post-translationally. Most information about such post-translational epitopes is available for glycosyl epitopes (consisting of sugars chains added to specific side chains in the protein), such as a structure called "Cross-reacting Carbohydrate Determinant, CCD, which will be discussed in more detail later. Other examples of post-translational modifications that may be part of an epitope are modifications of aminoacids by hydroxylation (hydroxyproline or hydroxylysine) or deamidation (formation of glutamate from glutamine) or phosphorylation.

Depending on their structure, two kinds of epitopes are often described. Some are termed conformational epitopes because their allergenicity (IgE-binding capacity) is tightly associated with the 3D structure of the protein. Once the protein is denatured so that the folding of the protein is disorganized, conformational epitopes are modified or destroyed. This is obviously the case with so-called discontinuous epitopes, composed of amino acids in different parts of the protein molecule brought together by the folding of the protein chain. Other epitopes are called linear epitopes because they are (largely) formed by a continuous sequence of amino acids on one peptide chain of the protein. The distinction between these two types of epitopes is not absolute, as many "linear" epitopes are recognized with much higher affinity in the folded protein compared to the isolated peptide.

Epitopes are widespread within the protein molecule. Similar to allergens, not all epitopes are recognised by all the individuals allergic to the protein, and some epitopes are immunodominant, i.e. recognized by the majority of these allergic individuals, while others are only recognised by a few allergic individuals. Thus, there is room for a tremendous variation between allergic individuals with regard to the precise targets of their allergic responses.

It has recently been shown that some epitopes may have a particular clinical significance depending on their structure and location within the molecule. Short linear IgE-binding epitopes which may be located in hydrophobic parts of allergenic proteins could be used as markers of a persistent food allergy, i.e. to milk and to peanut (Beyer et al., 2003; Chatchatee et al., 2001; Jarvinen et al., 2001). Such characterisation of epitopes, and particularly IgE-binding epitopes, may in the future provide information allowing the prediction of persistence and severity of clinical reactions.

Stability to processing and resistance to proteolysis by digestive enzymes have long been considered a general characteristic of food allergens (Astwood et al., 1996). However recent studies showed that the relationship between resistance to digestion and allergenicity is less clear cut than originally thought (Astwood et al., 1996; Diaz-Perales et al., 2003; Eiwegger et al., 2006; Fu et al., 2002; Vassilopoulou et al., 2006).

Allergenicity may be destroyed by processing (e.g. cooking) and/or digestion of the food. Alternatively, some epitopes may be unmasked and become available after denaturation or hydrolysis of the protein and neo-allergens or new immunoreactive structures may be created during processing including heat treatments (Berrens, 1996; Besler et al., 2001; Davis et al., 2001; Hansen et al., 2003; Maleki et al., 2000; Mondoulet et al., 2005; Nakamura et al., 2005).

In sum, there are 3 levels of structures (whole food, protein and epitopes) involved in the interaction with IgE antibodies and responsible for the allergenicity of a given whole food. Different epitopes and thereby different proteins and different foods will respond differently to processing and digestion. Therefore, depending on the immunodominance of the different epitopes in a particular individual, the effect of processing on food allergenicity may also differ between individual consumers (see Annex 2).

1.3. Mechanisms

Food allergy has to be understood as a disease where sensitization can occur either by cross-reactivity, i.e. first sensitization by a respiratory route to a common allergen (e.g. birch tree pollen allergens which have molecular homology with several fruit allergens), or primary sensitization through the gut. The second issue appears to be particularly important and relevant to potential food allergy triggered by genetically modified foods. The gastro-intestinal tract provides various types of physical barriers to allergens (Adel-Patient et al., 2008). Digestive enzymes and acid production in the stomach will denature allergens before they have a chance to gain access to the immune system. Factors in the gut mucus secretions such as secretory IgA as well as the physical barrier of the gut also constitute a protection against antigen penetration. Antigen recognition by the gut immune system depends then on protein size and on denaturation of major antigenic epitopes by digestion and proteolysis. These protecting mechanisms are at least in part, less effective during the first months of life, possibly explaining a window of susceptibility at this age for sensitization to common food allergens.

Although those non-specific mechanisms of protection are efficient, it should be kept in mind that in some cases protein digestion may not be necessarily complete. Protein digestion is strongly linked to transit rate and the nature of concomitant food intake which has an effect on both the transit and absorption rate of potential allergens. Besides, the wide use of gastric acid inhibitors in the general population significantly alters the conditions under which food proteins are digested. It should be noted that proteolytic cleavage of proteins into peptides does not necessarily abrogate immunogenicity, and this applies in particular to food antigens submitted to peptic digestion in the stomach. Furthermore, if an allergenic epitope is heat and acid resistant it will have a greater potential to sensitize even in adults. There is also the potential for sensitization to foods to occur by contact with inflammed skin and even by inhalation. Indeed recent studies have suggested that sensitization to food allergens is more likely to occur through inflammed skin, such as occurs in eczema, than via ingestion (Adel-Patient et al., 2007; Adel-Patient et al., 2008; Lack et al., 2003).

Allergens interact with the innate immune system via multiple mechanisms, which constitute the first event occurring when an allergen comes into contact with a living body. The innate immune system is highly conserved on an evolutionary basis. Exposure to an antigen at mucosal level results in a cascade of events such as activation of the innate immune system, but not specifically for allergenicity. The contact of a food antigen with the intestinal mucosa is normally followed by sampling carried out by dendritic cells extending pseudopods into the intestinal lumen.

When an antigen is processed by an antigen-presenting cell (APC) such as a dendritic cell for presentation into the context of major histocompatibility complex (MHC) class II determinants, the antigen is first uptaken by the late endosome, in which a number of modifications occur. These include proteolytic cleavage at very discrete sites, reduction of disulfide bonds and trimming, so as to generate a family of peptides of ± 20 aminoacids. These peptides compete for the binding to nascent MHC class II molecules and, are accommodated into the MHC cleft to ensure the best fit of aminoacids between the peptide and MHC class II residues. The result is that only a very limited number of T cell epitopes are actually presented at the APC surface, among a large series of potential T cell epitopes. By contrast, pre-formed peptides, or a protein having been naturally submitted to peptic digestion, can bind to MHC class II molecule with no requirement for processing, reduction or trimming off. This result in a much larger panel of epitopes presented, with capacity to activate a large diversity of T cells. This establishes a fundamental difference in the repertoire of T cells activated by processing a whole protein as opposed to peptides.

Under physiological conditions, dendritic cells process the antigen for presentation to T cells in the context of MHC-class II determinants, which results in tolerance (Strobel and Mowat, 2006). However, under other circumstances such as intestinal inflammation, or possibly in individuals with genetic susceptibility, tolerance is not established. This could result from antigen handling by activated dendritic cells or from the triggering of innate immunity mechanisms. The latter is made of a number of receptors located at the membrane of epithelial cells, such as the Toll-like receptors (TLRs), or inside epithelial cells, such as the NOD receptors. These receptors act as sensors to detect glycoproteins, polysaccharides, lipids or polynucleotides of diverse origin, including bacteria, virusses and parasites, which might be construed as danger signals. The signal resulting from receptor binding is followed by intracellular signalling required to elicit an adaptive immune response. There is therefore a first decision made at the level of the intestinal mucosa itself, whether tolerance or activation should occur. The synergy between innate immunity receptors and activation of DC is well established, since extension of DC pseudopods into the intestinal lumen depends on activation of TLRs. Yet, the precise mechanisms by which ligands of TLRs drive activation of DC remains poorly defined. A second decision will be made at the level of mesenteric lymph nodes, which is where the adaptive immune system is entering into action. Thus, DC instructed for tolerance induction or activation of an immune response migrate from the mucosa to mesenteric lymph nodes, where they encounter T cells. Depending on the message exchanged between DC and T cells in the context of antigen-specific, MHC-class II dependent cognate recognition, such T cells will be either activated or tolerized (Mowat, 2003). Activation through MHC-class II peptide presentation results in the recruitment of effector T cells (also known as effector T cells or Th cells) sharing the CD4 surface marker. These cells are therefore referred to as CD4+ Th cells (h stands for “helper”). Three types of CD4+ Th cells have been described: Th1, Th2 and Th17, whose function is explained below. Whenever tolerance is established and in particular oral tolerance, other CD4+ T cells can be recruited.

Oral tolerance can result from a number of mechanisms, classified as intrinsic or extrinsic. Intrinsic mechanisms involve induction of apoptosis (programmed cell death) and anergy. Apoptosis is induced by surface expression of receptors and ligands belonging to the family of “death receptors”, in particular Fas and its ligand, FasL. This leads to a cascade of intracellular events resulting in cell death. Anergy, which is lack of activation upon antigen recognition, is essentially induced in the absence of costimulatory signals, which in turn occurs in the absence of a danger signal.

Extrinsic mechanisms involve the induction of regulatory T cells, among which two distinct subsets can be recognized: Th3 cells exert their suppressive activity through the production of TGF-β while natural regulatory T cells exert a contact-dependent suppressive activity, notably through overexpression of CTLA-4. This surface molecule, when contacting its corresponding ligand on target cells, induces a catabolism of essential aminoacids which deprive target cells of vital nutrients.

Although the mesenteric lymph nodes serve a function of firewall to protect the systemic immune system from unnecessary activation or detrimental tolerance induction, it is noteworthy that when tolerance is established at the intestinal mucosal level, this is also translated into systemic tolerance. This seeming paradox most likely results from two distinct events. First, the passage of small amounts of antigens directly into the venous circulation towards the hepatic vein, short-circuiting lymphatic circulation drainage: antigens reaching the liver through the portal vein are known to induce tolerance instead of immune activation. Second, T cells educated to tolerance in the draining mesenteric lymph nodes migrate to other peripheral lymph nodes, resulting in lack of proliferation and absence of antibody production.

Three subsets of adaptive effector T cells, with different properties are currently recognised, although it is likely that other subtypes will be described in the future. The Th1 subset is characterized by expression of the transcription factor T-bet and a receptor for Interleukin-12 (IL-12). The translocation of T-bet to the nucleus triggers the transcription of cytokines characteristic of the Th1 subset, including IFN-γ and IL-2. The Th2 subset expresses the GATA3 transcription factor and a receptor for IL-4. Translocation of GATA3 to the nucleus activates the transcription of IL-4, IL-5 and IL-13. The Th17 subset is characterized by the RORgammat transcription factor and a receptor for IL-23. Not much is known about the mechanisms by which RORgammat induces transcription of cytokines, but is associated to production of IL-6, TNF-α and IL-17. The link between soluble cytokines and T cell subset is, however, not very strict as, for instance, a number of Th1 cells produce IL-5.

The differentiation of these T cell subsets depends on signals produced by antigen-processing cells, both soluble and surface-bound. For instance, Th1 cells differentiate upon binding of IL-12, Th2 upon binding of IL-4 and Th17 upon stimulation by IL-23, IL-6 and TGF-β. It should be noted that these subsets essentially exclude each other. IFN-γ produced by Th1 cells inhibits the maturation of Th2 cells. IL-4 blocks Th1 maturation and both IL-4 and IFN-γ block the maturation into Th17 cells.

Effector CD4+ T cells exist at different stages of activation and are amenable to regulation by intrinsic and extrinsic mechanisms, as described above, which might also lead to unresponsiveness through either physical or functional elimination of specific Th cells.

Each CD4+ effector T cells has been associated with pathology and, to some extent, specific isotype production. Crohn's disease represents a typical Th1-driven disease, while ulcerative colitis exemplifies Th2 pathology. Th17 cells are associated with diseases characterized by chronic inflammation, such as Crohn's and colitis, but also asthma. Th1 cells help in the production of immunoglobulins IgG1 and IgG3, while Th2 cells trigger the production of IgE and IgG4 antibodies. There is not yet an isotype associated with Th17.

Antigen presentation into the gut can result in activation or tolerance induction involving various types of cells pertaining to either the innate or adaptive immune system. When activation prevails several outcomes are possible, though these are not mutually exclusive:

- Activation of effector CD4+ T cells resulting in tissue inflammation and destruction in the absence of specific antibodies. T cell subsets producing inflammatory mediators such as IFN-γ (Th1 cells) or IL-6 (Th17 cells) predominate here.
- Activation of effector CD4+ T cells producing interleukins required for helping B cells in producing specific antibodies. Here the Th2 cells play a determinant role as an obligate condition for the production of IgE antibodies.

Antibodies participate in the pathology of allergy but do not necessarily represent a main component of it. Apart from the role of IgE antibodies, which is well established, i.e. by activating basophils and mast cells, resulting in the liberation of mediators such as histamine, other isotypes of antibody may participate in the different patologies of allergy but their role is not as well-established as for IgE.

Both innate and adaptative immune responses are tightly regulated. A failure within these regulatory mechanisms could lead the immune reaction towards antigens into becoming pathological. The mechanisms described above therefore can contribute to the pathogenic potential of a food antigen. An understanding of these cellular interactions occurring once the gut mucosa is in contact with a food antigen can facilitate the identification of early biomarkers of sensitization.

Natural resolution of food allergy occurs in up to 80% of milk allergy cases in early childhood in prospective studies (Host and Halken, 1990), egg (50%) and even peanut in 15% by the age of 3. There is currently no solid data to explain why and how the latter occurs but it is much less likely to occur beyond infancy. Many hypotheses, among which the maturation of the gut immune system, an increase in the number of regulatory T cells and modification of the gut flora elicited by qualitative changes in the diet have been pinpointed, but formal demonstration is still lacking.

As mentioned before, antibodies of other isotype than IgE, e.g. IgG, can be involved in the immune response induced by food antigens. IgG4 needs specific attention, the first reason is that the production of IgE is more closely linked to that of IgG4 than to that of other immunoglobulin isotypes, mostly because both IgE and IgG4 depend on IL-4 or IL-13 for the isotype switch. Since Th2 cells are important producers of these cytokines this is often referred to as a Th2-type immune response. Such immune responses are not so strongly stimulated (and often even markedly down-regulated) by microbial factors (such as endotoxins and other Toll receptor ligands) as the more conventional Th1-type immune responses. The second reason is that IgG4 antibodies to allergens have been described to be associated with allergic symptoms.

A relationship exists between IgE and IgG(4) from the immune regulatory point of view[8]. IgE producing B cells are descendants of IgM-producing B cells, either directly or indirectly. In the latter situation, the intermediate B cell is often an IgG4-producing B cell, but B cells producing other IgG isotypes may also act as intermediate for IgE producing B cells. An IgE response invariably includes production of other antibodies. The reverse is, obviously but still remarkably, not true: it is very common to find IgG1 antibodies in the absence of IgE antibodies. This indicates that the isotype switch in B cell differs between IgG1 and IgE (and/or that the expansion and differentiation of the switched B cells depend on factors linked to the isotype switch). The best-known factor is the type of cytokines produced by the Th cell: Th1 cytokines, particularly interferon gamma, are potent inducers of the isotype switch to IgG1 and suppress the IgE switch, whereas Th2 cytokines, particularly IL-4, induce the switch to IgE (and to IgG4). However, the selectivity of these switch factors is often concentration-dependent. Low concentrations of IL-4 have been found to induce IgG1 switching *in vitro* (Kotowicz and Callard, 1993). Also *in vivo*, an association is found between IgE responses and IgG responses, particularly for classical atopic allergens, such as pollen allergens and mite allergens. For these allergens it is rare to find IgG responses in the absence of IgE responses (Aalberse, 2006; Aalberse et al., 2009; Chapman and Platts-Mills, 1978; Lichtenstein et al., 1992; Platts-Mills, 1979; Thomas and Hales, 2007). This association, which is particularly convincingly demonstrable with assays that use purified allergens (rather than whole allergen extracts) and that preferentially measure high-affinity antibodies, is found not only for IgG4, but also for IgG1. Because of the association between IgE and IgG(4) responses, it has been suggested that IgG(4) responses might be involved in allergic symptoms.

The suspicion about the involvement of IgG4 in allergy in general, and food allergy in particular, was largely raised by two sets of observations. Firstly, the evidence provided by Pepys et al. (1979) that human IgG antibodies (IgG-STS, for short-term sensitizing IgG) could induce an allergic skin reaction in a passive transfer model in primates (the STS referred to the shorter duration of the sensitization: 4

[8] Note: in this document the isotype nomenclature refers to humans. The IgG isotypes in other species have often identical names without being homologous to the human isotype. It is particularly confusing that the mouse IgG1 isotype is NOT equivalent to human IgG1, but (to some degree, in immune regulatory context) equivalent to human IgG4.

hrs, compared to >24 hours for IgE) . Whereas the first data suggested that IgG-STS activity resided largely in the IgG4 fraction, subsequent experiments failed to substantiate this (Malley et al., 1985). However, some support came from *in vitro* studies, in which anti-IgG4 antibodies were found to activate basophils from some human subjects. However, claims regarding allergen-induced IgG4-dependent basophil activation could not be confirmed (Vantoorenenbergen and Aalberse, 1981). The anti-IgG4-induced basophil activation was probably due to IgG4 anti-IgE antibodies rather than to direct binding of IgG4 (Shakib and Smith, 1994).
The other argument for implying IgG4 antibodies as a significant factor contributing to allergic symptoms was the increased presence of allergen-specific IgG4 in allergic subjects (Halpern and Scott, 1988; Stanworth, 1987). Children with an atopic phenotype tend to have higher IgG antibodies to foods, also to foods that are well tolerated (Eysink et al., 1999), possibly due to a defective mucosal barrier function or to a general increased immune reactivity (Salvaggio et al., 1969). In the specific case of IgG4, the association between IgE and IgG4 is likely to reflect also the IL-4 dependency of both IgE and IgG4 production. A main obstacle in accepting IgG4 as a contributing factor in allergen-induced symptoms was the presence of often large amounts of allergen-specific IgG4 in the absence of symptoms. A striking example is provided by the beekeepers, who often have huge levels of venom-specific IgG4 without any symptoms upon being stung and a negative skin test. Also the positive association between IgG4 to airborne allergens and protective effects of allergen-specific immunotherapy are more supportive of a protective than a pathogenic role of allergen-specific IgG4.

In conclusion, there is no convincing evidence or mechanism to support the notion of IgG4 as a sensitizing antibody. IgG antibody levels (including IgG4 antibodies) to foods tend statistically to be slightly higher more prevalent in atopic subjects than in controls, but such antibodies (including high levels) are too common in the food-tolerant population to be of diagnostic use. The induction of IgG antibodies following the introduction of a novel protein into the diet can not be considered as an abnormal response.

1.4. Prevalence

Food allergies are a commonly suspected problem as up to 1/3 of a non-selected population believes they suffer from food allergy (Sloan and Powers, 1986; Young et al., 1994). Many individuals who believe they are allergic to foods do not have a reproducible response on double blind food challenge. They are described as having food aversion or psychological intolerance which is a common phenomenon. This emphasizes the need for accurate assessment and diagnosis because food avoidance in the mistaken belief about allergy is not without potential complications, including nutritional imbalance and reduced quality of life. However, not all adverse reactions to food are attributable to food allergy, as by definition, the immune system has to be involved in pathogenesis of the disease (Johansson et al., 2001), other reproducible responses may be due to errors in digestion and/or metabolism (e.g. lactose intolerance). Epidemiological studies pinpointing the right diagnosis by standardized food challenges have highlighted a prevalence of 5 to 8 % in infants and young children and less than 5 % in adults (Bock, 1987; Jansen et al., 1994). Most epidemiological studies were designed to detect IgE-mediated food allergy but few data are available on non IgE-mediated food allergies. In infants, a prospective study on cow's milk allergy reported approximately half of them (corresponding to approx 1-2%) being non IgE-mediated (Host and Halken, 1990). In addition, data on non IgE-mediated food allergy can be extrapolated from population-based studies, suggesting that if adverse reactions to foods are suspected in up to 30% of the population, IgE-mediated food allergy can only be diagnosed in up to 5 % of the population, the rest being intolerances or non IgE-mediated food allergy (Sloan and Powers, 1986; Young et al., 1994).
Almost all edible proteins have been described either in large series or in case reports as a possible food allergen. However, a relatively short list of foods is encountered as common food allergens around the world. Milk is the prototypic food allergen that was described already by physicians in ancient Greece. In addition, egg, peanuts and nuts, fish and crustacean, various grains, vegetables and fruits are commonly identified (Bock, 1987; Eigenmann et al., 1998) see Directive 2006/142/EC for labelling purposes (EC, 2006). There is a clear link between cultural eating habits and the foods most

frequently encountered, e.g. in maritime countries, fish allergy is rather common, while South Korea has a high number of buckwheat allergic individuals, with buckwheat noodles being consumed in large amounts from early in life. As with all allergic diseases there is good evidence that food allergy prevalence has been increasing over the last 3-4 decades initially in developed countries but now also in the developing world. The geographical distribution of susceptibility to allergy has been associated with affluence, which has been explained as being due to reduced exposure to infections in early life. However, many other environmental influences are likely to be involved. So far, no scientific report has described allergic individuals with clinical reaction specific to GM food. However, it can be assumed that for crops such as soy that are consumed in relatively high quantities, including GM plants, allergic individuals react to constitutive proteins of the food.
No information is available on whether or not an extensive cultivation and/or use of GM crops known to be allergenic e.g. soy, has changed the prevalence of inhalant or food allergy in the exposed populations.

1.5. Clinical pictures

Immune mediated adverse reactions to foods must be distinguished from intolerances due to errors in digestive or metabolic processing of the food. The first are most commonly associated with IgE generation but can sometimes occur in the absence of IgE, which is described as non-IgE-mediated food allergy. It may then either be mediated by activation of eosinophils which evolves between 4 and 24 hours after exposure to the food or activated T-lymphocytes which takes several days to develop. However, some overlap might exist between the two classical forms, especially food allergies in the gastrointestinal (GI) tract with eosinophilic infiltration of mucosa where a subset of allergic individuals have positive IgE tests (Fig. 1). In describing the range of clinical features associated with food allergy it is important to be aware that there can be many other causes of the same presentation.

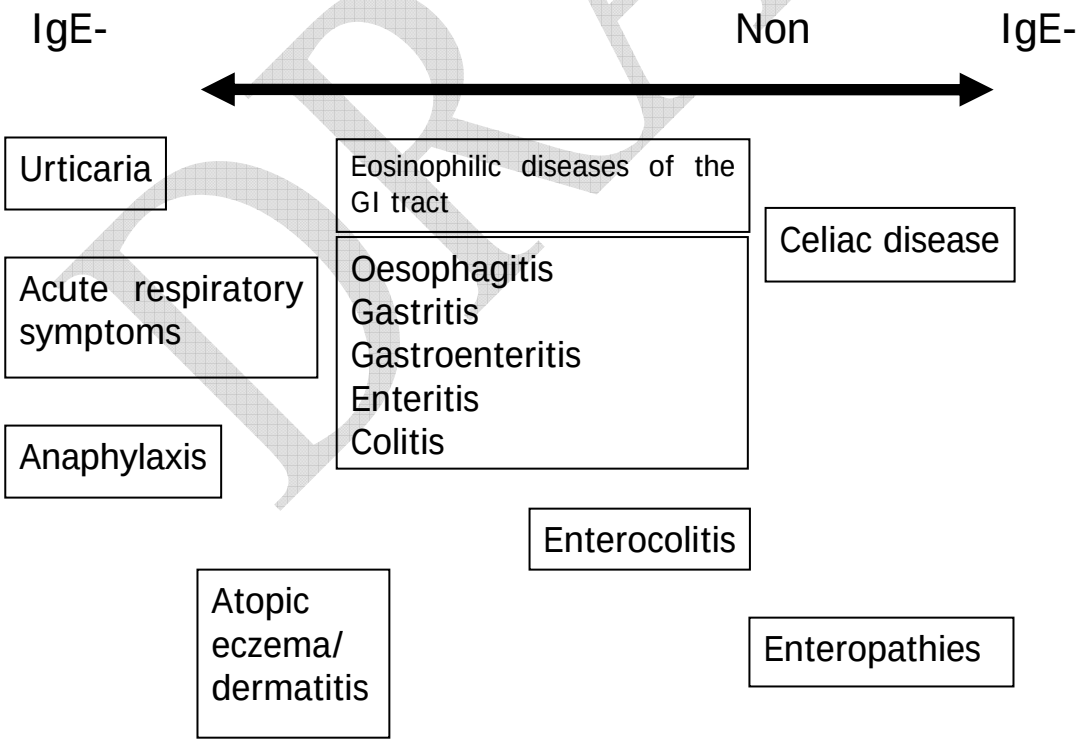

Figure 1: Clinical manifestations of food allergy classified according to the pathogenesis of the disease. Adapted from Rothenberg (2004).

IgE-mediated food allergy can present with clinical signs limited to one system or involving several systems (anaphylaxis). Skin is probably one of the most commonly involved systems in food allergy. Urticaria, sometimes known as hives, is a common reaction of mild food allergy as an isolated symptom, or in combination with other manifestations in anaphylaxis. Urticaria usually manifests within minutes to 1 to 2 hours after ingestion of the food. Virtually, all foods eliciting IgE-mediated food allergy can provoke urticaria. The skin eruption is very similar to that seen after a nettle sting and is intensely itchy. It is a consequence of the release of mast cell mediators such as histamine. These irritate sensory nerves, cause dilatation of blood vessels and leakage of fluid from the circulation into the superficial layers of the skin. Some allergic individuals might also present with a mixed clinical picture of urticaria and angio-edema, the latter being a reaction in the deeper layers of tissue below the skin, with swelling most characteristically round the eyes and lips. If it affects the tissues in the throat swelling may obstruct breathing and constitute a life threatening manifestation (laryngeal oedema). Atopic eczema/dermatitis is another common manifestation of food allergy. Food allergy associated flares of atopic dermatitis are seen most commonly in young children. Up to 40% of young children with moderate to severe atopic dermatitis can have an associated food allergy (Burks et al., 1998; Eigenmann and Calza, 2000; Eigenmann et al., 1998). However, food allergy is a less common association with eczema in older children and adults. The respiratory system might be involved leading to asthmatic symptoms and anaphylaxis. Acute rhinitis or broncho-constriction can be of rapid onset. Signs of asthma are of particular importance for the prognosis of food allergy. Indeed it has been shown in several studies that near fatal and fatal reactions to foods are determined by pre-existing asthma (Bock et al., 2001; Sampson and McCaskill, 1985). Respiratory symptoms are present without any other clinical symptoms in about 20 % of allergic individuals (Sampson and McCaskill, 1985). It is believed that isolated and chronic recurrent respiratory symptoms are uncommonly caused by food allergy alone. However, life threatening events can occur without prominent respiratory symptoms if massive basophil activation leads to extensive leakage of fluid from the circulation. This produces a drastic fall in circulating volume and blood pressure leading to collapse and cardiac arrest. Anaphylaxis is the word used to describe this life threatening reaction. Symptoms of the gastro-intestinal tract involvement including vomiting or diarrhea are often seen within the clinical picture of anaphylaxis. If present without any other clinical signs, these forms of food allergy are more commonly of the non IgE-mediated type. Finally, oral pruritus is one of the most common forms of IgE-mediated food allergy. It is related to oral allergy syndrome, and this manifestation of food allergy is mostly seen in adolescents and adults and is linked to a primary sensitization to a cross-reacting pollen protein (e.g. Bet v 1) which has sequence homology with crude apple and other tree fruit proteins (Amlot et al., 1987; Ortolani et al., 1988).

Clinical manifestation can be provoked by the same foods as those involved in IgE-mediated food allergy but through different mechanisms. In young children, the diagnosis of non-IgE-mediated food allergy is mostly made by the history, showing a characteristic constellation of symptoms (Sampson and Anderson, 2000). Food protein-induced enteropathies are diagnosed in infants usually less than 6 months old. Classically it presents as profuse and repeated vomiting after a symptom free interval of 2 to up to 6 hours after ingestion of the food (Nowak-Wegrzyn et al., 2003; Powell, 1978; Sicherer et al., 1998). The offending food is most often milk but can also be soy, egg or other solid foods. The disease is self-limited in time and most often disappears after 2 to 3 years of a food elimination diet. Milk induced procto-colitis is a disease in which the child presents with isolated fresh blood in the faeces. It is seen in a few weeks old breast-feed infants and sometimes in bottle fed babies (Belli et al., 1994; Lake et al., 1982) and it disappears after the milk has been removed from diet.

Food-induced enteropathy is a pleomorphic disease in which an immunological mechanism is suspected but not always proven (Ford et al., 1983). However, where gut biopsies have been performed eosinophil or lymphocyte infiltration and occasionally subtotal villous atrophy (similarly to coeliac disease) is seen. Adults and children with chronic diarrhea or vomiting, children with failure to thrive or other non-specific GI symptoms are most often diagnosed with this condition. Food induced enteropathy may be self-limiting and resolve after one or two years but on occasions can be a persistent problem. Eosinophilic disease of the gastro-intestinal track was first described only a few

decades ago. There is a clear trend towards increase of prevalence of these diseases. They can present with eosinophilic infiltration of the lower esophagus (Kelly et al., 1995; Naylor, 1990) but this form of inflammation can be present at any level in the gastro-intestinal tract. Over the last few years an ever increasing range of gut motility disorders have been described in association with eosinophilic inflammation. In children and adolescents, this form of eosophilic disease is more often related to a specific food. The classical clinical picture is that of untreatable reflux with hyper-secretion of mucus and abdominal pain. However, chronic diarrhoea, constipation, abdominal pain, failure to thrive in children and weight loss in adults can be associated.

Adults complaining of isolated gastro-intestinal symptoms may suffer from food aversion, intolerance or allergy. A recent cross-sectional study performed in Berlin showed an overall self-reported prevalence of adverse reactions to foods of 34.9%. Double-blind, placebo-controlled food challenges identified reproducible adverse reactions to foods in 3.7% of the adult population, of which 1.1% were non-IgE-mediated (Zuberbier et al., 2004). Other epidemiological studies in the UK and in the Netherlands have shown similar results (Jansen et al., 1994; Young et al., 1994). A specific clinical picture of non-IgE-mediated food allergy in adults is represented by eosinophilic esophagitis, a condition differing from the pediatric form described above by the lower rate of associated food allergy and by dysphagia being the leading symptom (Straumann et al., 2003). Animal models for eosinophilic disease of the gut have been established and should allow further mechanistic as well as therapeutic studies of this type of diseases (Mishra et al., 2001).

Coeliac disease is well characterised by the clear involvement of food, i.e. gluten present in wheat and related cereals, in the pathogenesis of an immune reaction in absence of IgE antibodies. The modification of gluten-derived peptides by tissue transglutaminase-mediated deamidation of glutamine residues increases the binding affinity to particular alleles of the major class II histocompatibility complex, i.e. HLA-DQ-2.5 and HLA-DQ-8, the expression of which is strongly associated with coeliac disease. Effector T cell reactivity is thereby increased. This mechanism serves as a basis to define coeliac disease as an auto-immune disease associated with local gut epithelium inflammation. The prevalence of the condition in communities varies with the frequency of the tissue types and in Europe is estimated to be between 0.2 and 1.2%. The proteins responsible for the response are prolamins, rich in proline and glutamine (gliadin from wheat, hordein from barley and sacalin from rye). There is some dispute as to whether the prolamin, avenin from oats may also cause the disease. Dietary exposure to the proteins, sometimes facilitated by simultaneous gut infection with rotavirus or adenovirus leads to the production of tissue transglutaminase (tTG) auto-antibodies. tTG participates in the processing of gluten and there is a suggestion that there is an innate immune response to the gliadin. This results in a lymphocyte mediated inflammatory response with loss of the villus (folding) architecture of the small bowel wall, together with mucus gland hypertrophy. This results in mal-absorption of nutrients with resulting iron deficiency anaemia, failure to thrive in infants and weight loss in adults. The failure to absorb fat sometimes results in offensive diarrhoea known as steatorrhoea, abdominal bloating and pain. Many allergic individuals particularly children with coeliac disease recovered once gluten is excluded from the diet. Several types of animal models with disease expression mimicking coeliac diseases have been established. They include MHC II transgenic mice (Mangalam et al., 2008), as well as gluten-sensitivity animal models in various types of animals including non human primates (Bethune et al., 2008).

The treatment is primarily life-long avoidance of gluten, and for the rare refractory case immune modulation with steroids or other immune suppressants.

1.6. Diagnostic procedures

The diagnosis of food allergy is largely based on the clinical history. In fact, a history of repeated ingestion leading to rapid occurrence of typical symptoms is highly suggestive of an adverse reaction to food. Furthermore, the clinical picture as described in the section above will increase the degree of suspicion for a food allergy. In suspected IgE-mediated food allergy, the diagnosis will rely on positive skin prick testing by commercial food extracts or native food extracts. Furthermore, serum specific IgE can be measured to most potential food allergens. Interpretation of positive tests can be

difficult as a positive test can also indicate presence of a sensitization without clinical relevance. Various studies have provided cut-off levels for specific foods (Boyano-Martinez et al., 2002; Celik-Bilgili et al., 2005; Osterballe and Bindslev-Jensen, 2003; Sampson, 2001). It is now well established that these cut off levels depend on the food, the type of symptoms, as well as the population age. Thus cut off levels should be interpreted with caution (Eigenmann, 2005).

It has been suggested that an atopy patch test can aid diagnosis in non-IgE mediated food allergy. The food is applied to a small patch of skin under an occlusive dressing for 48 hours. The skin is examined for a reaction 24 hours after the dressing has been removed. A positive response has a characteristic eczematous appearance. However, techniques have not been standardised and this approach has not yet undergone sufficient validation to be considered a routine diagnostic test.

Diagnosis of non-IgE-mediated reactions mostly relies on the clinical history as described previously. Few reports have shown that activation of peripheral blood mononuclear cells by antigen can be of some help for the diagnosis. The diagnosis of coeliac disease is based on detection of circulating IgG and IgA tissue transglutaminase antibodies and the presence of villus atrophy and lymphocyte infiltration in a small bowel biopsy. More recent developed diagnostic tests included leucotriene release assays or CD63 activation or basophilic histamine release. However, these procedures are not yet clinically validated.

Thus, the diagnosis of food allergy has to rely on resolution following withdrawal of the food(s) and the reoccurrence of symptoms during food challenges. Standardized food challenge protocols have been developed for the different clinical pictures. They are currently the gold standard for the diagnosis of food allergy (Bindslev-Jensen et al., 2004; Bock et al., 1988; Niggemann et al., 1994), but not without problems. During challenges factors which might otherwise adjuvantise a response, such as concurrent infection, heavy exercise etc. are eliminated. Furthermore, in order to disguise the taste of the food in a double blind procedure the immediate effects on the buccal mucosa (lining of the mouth) are lost. Assessing the response can also be difficult if only subjective symptoms occur.
Double-blind, placebo-controlled food challenges (DBPCFC) have been elaborated firstly to diagnose food allergy in children with atopic dermatitis (Sampson, 1983). However, this procedure has quickly evolved to become the standard diagnostic test for food allergy in research protocols as well as in many clinical situations. If performed routinely in centres with well trained staff, these procedures can be easily performed in one- or two- day protocols. Although most centres use slightly different procedures, which need to be adapted to each allergic individual's own situation, wide efforts have been recently undertaken to standardize the procedure (Bindslev-Jensen et al., 2004; Bock et al., 1988; Niggemann et al., 1994; Sampson, 1983).
In particular issues related to clinical assessment of foods derived from GMO designed to constitute a hypoallergenic food, for example by decreasing the expression of endogenous allergens, the need for DBPCFC could be considered, although this would rather be a substantiation of health claims than a safety assessment.

1.7. What happens when a new food is introduced into the diet?

For as long as we know history, the diet of the population has been changing more or less rapidly with new foods being taken into use. New allergens certainly have been introduced many times. Some fifty years ago kiwi fruit was introduced from New Zealand into California and USA in general, and later into Europe. During the last decade, the use of lupin flour has become much more common than before in Europe and has spread to several countries where lupin never before was part of the diet.

What will happen after introduction of a new or newly allergenic food, will depend on whether it elicits clinical reactions by cross-allergenicity, or whether clinical reactions depend on primary sensitization to the new allergen. Reactions due to cross-reactivity will occur as a “first wave” as soon as the food has been introduced, while reactions depending on primary sensitization will come later as a “second wave”. Totally novel allergens without cross-reactivity may only manifest themselves

following primary sensitization. The two types of reactions may differ clinically, and reactions due to primary sensitization generally tend to be more severe than reactions caused by cross-allergenicity.

Thus, after introduction of a new or newly allergenic (modified) food, there will first be clinical reactions due to cross-allergenicity (if any) that tend to appear early after introduction of the food, because the consumers are already allergic (Fig. 1). These reactions will take place from day 1 after introduction of the food, and their true frequency will only depend on the extent to which the food is consumed. However, the observed frequency will tend to be low in the beginning and increase as awareness of the problem increases among the population and among health care providers. These reactions will schematically tend to be less severe because they are due to cross-reactivity. Later, because primary sensitization has first to take place, reactions caused by primary sensitization to the new allergen will gradually appear (Fig. 2). The true frequency will probably increase rather slowly, because different individuals are likely to be sensitized at different speeds depending on individual factors and patterns of food consumption. Also, it is likely that the observed frequency will develop slowly, because recognition of the allergenic properties of the food and awareness of the problem most likely will take a long time to develop.

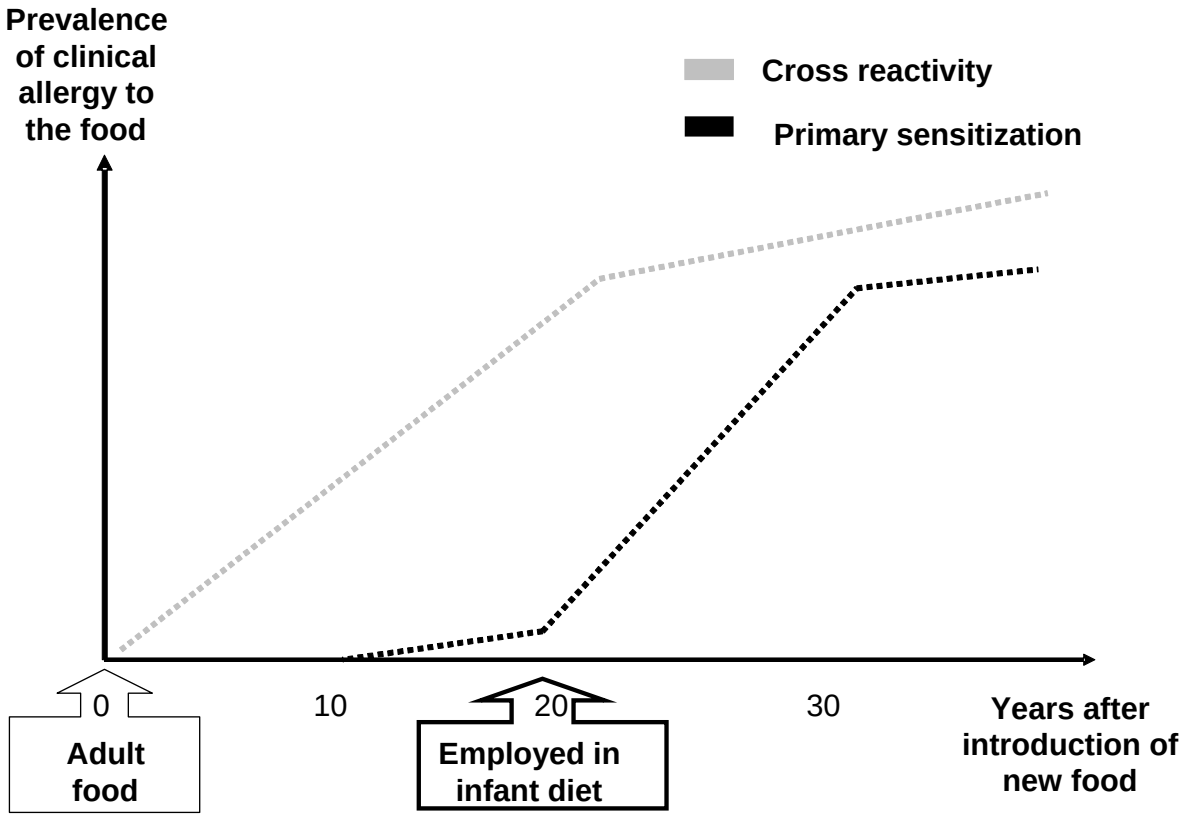

Figure 2: Schematic illustration of how, after introduction of Kiwi fruit into UK in 1970 clinical allergic reactions to kiwi exposure appeared in the population. Initially, it was predominantly eaten by adults and most who developed allergic reactions did so as a consequence of cross reactivity in those with pre-existing pollen and latex allergy. Most adults had relatively mild symptoms. Only after around 20 years did kiwi begin to appear in infant feeds. This was followed by increasing numbers of children presenting with allergic reactions to kiwi with a significant percentage having severe manifestations including anaphylaxis (Lucas et al., 2004).

On most of the European market, lupin is in effect a new allergenic food, and the picture of its allergenicity in the population is still evolving. Severe food allergic reactions caused by lupin appeared fairly soon after lupin flour was used to fortify wheat flour, and most of the food allergic reactions to lupin observed so far have apparently been caused by cross-allergenicity with peanut (Faeste et al., 2004; Moneret-Vautrin et al., 1999). Many of the reactions have been relatively severe (Radcliffe et al., 2005; Smith et al., 2004; Wuthrich, 2008). The extent of clinically important cross-allergenicity between lupin and peanut has been reported and is still a matter of debate and further investigations are needed (Lindvik et al., 2008). Primary lupin allergy has been reported (Faeste et al., 2004; Peeters et al., 2007; Quaresma et al., 2007; Wassenberg and Hofer, 2007; Wuthrich, 2008), but it remains to be seen whether primary lupin allergy will develop into a significant clinical problem. A difficulty hampering a good understanding of the development of food allergy to lupin after its introduction into the general diet in Europe, is the lack of information about the amount of lupin consumed at the national and individual levels. An early warning sign that lupin was allergenic was that people working with the lupin sometimes developed asthmatic reactions due to inhalation of the flour.

Thus, we can learn from history that if a novel food product cross-reactive with a common food allergen were introduced into the diet, clinical food allergic reactions would be expected to appear almost instantly. The further development of reaction frequency would depend essentially on the extent of product intake and exposure. If cross-reactivity were with a less common pre-existing food allergen, reactions still would occur early but the frequency would develop to lower levels. Recognition of cross-reactivity with a common allergen is likely to be rapid given the awareness of the potential problem among the population and caregivers whilst the recognition of cross-reactivity with a less common allergen is likely to be slow and less clear. In contrast, if reactivity to the GM food would depend on primary sensitization, it would take months and possibly years before a significant number of clinical reactions appeared, and the recognition of the problem might develop very late and slowly. Moreover, primary sensitization is more likely to occur early in life when the immune system is immature and become apparent when the food is eaten by infants.

1.8. Specific assessment for children

Food allergy in childhood is clinically different from adult food allergy. In infants and children, symptoms consist of skin reactions (i.e. urticaria and flares of atopic dermatitis), as well as digestive and/or respiratory reaction, while in adults symptoms are mostly related to foods cross-reacting with pollen proteins (i.e. oral allergy syndrome). Primary sensitization to the incriminated foods largely explains the different type of clinical response. In infants, primary sensitization occur through the gut or also possibly prior to birth. Primary sensitization in the gut of infants might be favoured by the immaturity of the local immunity and incomplete barrier function of the intestinal gut mucosa as well as incomplete protein degradation by pepsin in the stomach due to a gastric pH above values seen in adults. It has been well demonstrated that gastric acid secretion attains adult levels only after one month of age (Hyman et al., 1985). For instance, it has been shown that at the pH in the stomach of an infant the major allergenic proteins in kiwi fruit are not digested, thus increasing the potential for primary sensitization. This does not occur in older children and adults with more acidic conditions in the stomach (Lucas et al., 2008). The potential of allergy sensitization in a gastric environment with increased pH is further corroborated by the increased risk for allergic sensitization found in allergic individuals treated with anti-acid medications (Lucas et al., 2008; Untersmayr and Jensen-Jarolim, 2008).

In adults, primary sensitization can occur through the gut, but mostly by cross-reaction between fruit and legume proteins with common pollens (e.g. apple and birch pollens). This is exemplified by reactions to kiwi fruit which have been seen progressively more frequently over the last 30 years in Europe. The commonest adult manifestation is with minor symptoms of the oral allergy syndrome. As it was more recently manifested in young children severe generalised allergic reactions including anaphylaxis occurred. The presumption is that the children were primarily sensitized with the potential

for severe reaction while adults had cross-reactivity with prior pollen allergy and consequently milder responses.

1.9. Other considerations

1.9.1. Immunogenicity and Adjuvanticity

Immunogenicity is the capacity of a proteins to induce an immune response and particularly activation of T cell lymphocyte subsets and the production of antibodies of different isotypes in humans and animals after exposure under appropriate conditions. Peptide fragments generated after proteolytic cleavage of the protein may retain (part of) the immunogenicity or acquire new immunogenic properties (see Annex 1.3).

Adjuvants (from Latin adjuvare, "to help") are substances that, when co-administered with an antigen, increase the immune response to that antigen that is to say that they increase the immunogenicity of a protein. By modifying the microenvironment in which antigen-presenting cells (APC) are active, an adjuvant activates the innate immune system in order to increase the efficiency of antigen presentation to T lymphocytes. This can be achieved by many different pathways, including increased surface expression of MHC molecules, co-stimulatory molecules, adhesion molecules, as well as production of soluble cytokines such as IL-12.

Adjuvants activate APC by triggering signaling as a result of recognition of receptors of the innate immune system, Toll-like receptors (TLRs) and NOD receptors, in particular. Aluminium salts exert their adjuvant effect by a combination of depot effect and activiation of the inflammasome through caspase 1 and the release of IL-1b. The hallmark of such receptor recognition is the activation of the transcription factor NF-kB and its translocation to the nucleus.

The adjuvant itself often is not immunogenic but injection of some adjuvant such as Freund's complete Adjuvant (CFA) or Lipopolysaccharides (LPS) alone can profoundly affect the immunological status of an animal. Adjuvants can activate NK and NKT cells resulting in increased pathogenicity in absence of any antigen. Adjuvants can elicit strong regulatory T cell responses. In some cases it may be that some proteins, e.g. choleratoxin are potent adjuvants and are also antigens that may trigger an immune response against themselves (Holmgren et al., 1994; Lebens and Holmgren, 1994; Moreno-Fierros et al., 2000; Pizza et al., 2001). The immune response facilitated by an adjuvant may sometimes be predominantly of one particular type qualitatively, e.g. a Th1-response or a Th2-response, in which case the adjuvant would be called a "Th1-adjuvant" or a "Th2-adjuvant", respectively. The qualitative polarization of the immune response caused by an adjuvant may be more or less marked, but may sometimes be strong and may override a pre-existing immune response. For example, CpG olignucleotides (components of bacterial DNA) are strong Th1 adjuvants, and may overrun a pre-existing Th2-type response against an antigen (Senti et al., 2009; Serebrisky et al., 2000) and lead to a strong Th1-type response against that antigen. Many vaccines contain an adjuvant to obtain the desired immune response quantitatively as well as qualitatively. In the field of vaccinology, much work is focused on the development of safe and effective adjuvants (Chiarella et al., 2007; Guy, 2007; Kwissa et al., 2007).

The term "adjuvant" is correspondingly used in environmental medicine to characterize factors that increase the development of immune responses, sometimes causing adverse effects ("environmental adjuvants"). For example, tobacco smoke is an adjuvant for allergy development (Nielsen et al., 2005), and there is an abundant literature on the adjuvant effects of diesel exhaust particles in relation to the increase of specific IgE production leading the development of allergy (Fernvik et al., 2002; Lovik et al., 1997; Samuelsen et al., 2008). The adjuvant effect of diesel exhaust particles has been found to be mediated both by adsorbed chemical substances and by the carbonaceous particle core itself (Granum and Lovik, 2002). Further, chemically inert particles in the fine and ultrafine (nano) size range have been found to have an adjuvant effect on antibody production (Granum et al., 2001a; Lovik et al.,

1997; Nygaard et al., 2009; Nygaard et al., 2004). Thus, it appears that physical as well as chemical properties may determine adjuvanticity (Granum and Lovik, 2002).

The antigen and the adjuvant have to be injected or applied together to achieve maximal adjuvant effect (Granum et al., 2001b). The precise mechanism underlying adjuvant activity is not known, and different mechanisms may contribute. One probable main type of mechanism is the focussing of antigen at a site where lymphocytes can react to it, and maintenance of antigen exposure at this site ("depot" effect). The other main type of mechanism is alteration of cellular functions (e.g. cytokine production) leading to a stronger immune response. The adjuvant may increase antigen capture and processing by antigen-presenting dendritic cells, and promote migration of dendritic cells to the lymph node. Released stress molecules may be of importance. Generally, it is thought that the capacity to induce local inflammation is important, because this will increase the local availability of both lymphocytes and antigen-presenting cells in a setting of cellular activation. Adjuvants may represent a "danger signal" to the immune system (Gallucci and Matzinger, 2001), and interact with pattern recognition receptors of dendritic cells, in particular Toll-like receptors (TLRs) and NOD receptors (Franchi et al., 2009; Lahiri et al., 2008; Trinchieri and Sher, 2007). Thereby the adjuvants may influence the magnitude and quality of the immune response by increasing the efficiency of antigen presentation to T lymphocytes. This can be achieved by different pathways, including increased surface expression of MHC molecules, co-stimulatory molecules, adhesion molecules, as well as production of soluble cytokines such as IL-12. A hallmark of pattern recognition receptor stimulation is the activation of transcription factor NF-kB and its translocation to the nucleus.
In addition to substances having a direct adjuvant effect, other substances may exert an indirect adjuvant effect by reducing food protein enzymatic degradation (Untersmayr et al., 2003) or by increasing antigen uptake (e.g. saponins in foods) (Maharaj et al., 1986).
It is nowadays feasible to detect *in vitro* an adjuvant activity by assessing, in cultures of APC, a few of the multiple parameters that characterize activation, both at genomic and phenotypic levels. The formation of an inflammasome within cells can be evaluated for instance by detecting the activity of caspases (Franchi et al., 2009). The precise pathway through which a putative adjuvant is exerting its activity can be determined using cells from genetically-modified animals (mostly knocked-out mice), silencing RNA and neutralizing antibodies. Such experiments are becoming commonly used. They may not detect all mechanisms for adjuvanticity/ types of adjuvants nor allow to distinguish IgE and cytotoxic adjuvanticity from IgA/IgG/IgM but they would provide useful information for assessing the adjuvant effect of newly expressed proteins or any product derived from GMOs (Marrack et al., 2009). Such *in vitro* models can be used in association with animal models (see below).

There are several classes of adjuvants.

- Mineral oil emulsions with or without killed mycobacteria (Freunds complete and incomplete adjuant, respectively) have been much used in animals but can not be used in man (Claassen et al., 1992; Stills, 2005).
- Some cytokines may be used as adjuvants, and administration of cytokines such as IL-2, GM-CSF and IL-12 has in human volunteers and animals been found to enhance the development of a mucosal immune response by an antigen (Toka et al., 2004; Wright et al., 2008).
- So-called CpG motifs, sequences about 20-fold more common in bacterial than in mammalian DNA, are effective Th1- and cytotoxic immune response adjuvants in mice, but results have not been found directly transferable to humans and other base sequences are needed for optimal function in humans (Gupta and Cooper, 2008; Klinman, 2006).
- Alum, a gel composed of aluminium potassium sulphate, aluminium hydroxide or aluminium phosphate also refererred to as alum, was one of the first adjuvants used and is still the only one commonly used in humans. It is extensively used e.g. in vaccines for children. The mechanism of action is beginning to be understood (Kool et al., 2008; Lambrecht et al., 2009), Alum promotes a Th2-biased antibody response (Lindblad, 2004) and is less good at supporting a cell-mediated immune response.
- Finally, some bacterial toxins or their components and modified versions have been found to be potent adjuvants in experimental animals and in humans, e.g. cholera toxin and heat-labile

enterotoxin (HLT) from *E. coli*. To produce a molecule that is no longer toxic but still has adjuvant activity, modified versions of cholera toxin may be made by point mutation or removal of the A chain (Pizza et al., 2001; Sanchez and Holmgren, 2008; Vazquez et al., 1999; Zhou et al., 2009).

While adjuvanticity, in particular for the IgG, IgM and IgA isotypes, is beneficial in some settings like in vaccines, IgE adjuvant activity will increase the risk of allergy. With vaccines, this has in practice turned out not to be a problem of practical significance. In contrast, in relation to respiratory allergy, exposure of the mucosa of the airways to environmental adjuvants like tobacco smoke (Nielsen et al., 2005) and diesel exhaust particles (Diaz-Sanchez et al., 1999; Lovik et al., 1997) has been convincingly demonstrated to increase the development of IgE-mediated allergy. Interestingly, it has also been demonstrated that a very important cause of allergy, pollen grains, in addition to their protein allergens carry with them substances that function as adjuvants. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization (Traidl-Hoffmann et al., 2005). The combined exposure to pollen allergens and substances with pronounced adjuvant activity may, at least in part, explain the high prevalence of pollen allergy and points to the importance of adjuvants in a normal physiological mucosal exposure setting when it comes to IgE-mediated allergy. Also in the intestines, adjuvant-facilitated IgE responses in experimental systems lead to food allergy and therefore clearly may cause adverse effects (Li et al., 2000; Untersmayr et al., 2003), and the same appears to be the case in man (Scholl et al., 2005; Untersmayr et al., 2005). An adjuvant-induced increase in production of antibodies of the IgA isotype would supposedly protect the mucosa both in the airways and in the intestines. It is less clear whether an increased production of IgG subclasses because of adjuvant activity would be beneficial or might lead to adverse health effects. An IgG response has a protective role against infections in the airways, and an IgG response against food proteins appears to be a normal response in the gut. However, whereas in the lung a strong IgG response against innocuous antigens may cause severe symptoms in so-called extrinsic allergic alveolitis (farmer's lung, pidgeon-breeder's lung, etc), it is still unclear whether an adjuvant-enhanced strong IgG response against food components would cause adverse effects. Also, it is still controversial whether an induction of strong Th1 immunity would protect against Th2-dependent allergic responses or lead to inflammatory conditions. Immunization experiments in rodents with subcutaneous injection may serve for screening and hazard identification (Lovik et al., 2007). However, considering the presumably most relevant route of exposure, peroral immunization models in mice have also been developed at the experimental level (see section on *in vivo* models; (Brunner et al., 2009; Li et al., 2000; Untersmayr et al., 2003; Vinje et al., 2009). As previously mentioned, an increased IgE response effect is strongly linked to allergy development and should be considered an adverse effect, whereas an IgA reponse is beneficial and, at least, not adverse. With regard to IgM and IgG, it is still very speculative whether an increased response to food allergens may cause adverse effects. Considering the fact that no condition is known in which IgG and IgM antibodies are the primary cause of adverse effects in the intestines, an IgG or IgM adjuvant effect not accompanied by increased IgE production probably should not be considered an adverse effect. However, further research addressing this issue should be undertaken.

In relation to foods, a food component with (strong) adjuvant activity would be expected to increase the development of allergy not only against that particular food. Instead, the effect would probably be stronger against other foods eaten concomitantly, if these were strong allergens (like milk, eggs, nuts, peanuts, etc.).

1.9.2. Food allergy in food-producing and companion animals.

Food allergy in farm animals has been reported in veal calves from dairy herds and piglets after weaning (review by Dreau and Lalles, 1999). Such food adverse reactions developed in baby calves when milk protein was replaced in milk formulas by insufficiently processed plant proteins, including sources from soya bean (*Glycine max.*) and field pea (*Pisum sativum*). The process of early weaning

onto non-milk dry diets leads to adverse reactions to food in baby pigs. In both animal species, gastrointestinal signs of reactions to food are the most common. In farm-reared fish, including salmon and rainbow trout many attempts have been made for replacing fish protein with plant proteins, from soya bean mostly. Food-mediated disorders have long been suspected based on gastrointestinal tissue alterations with such vegetal-based diets. However, little evidence exists presently to indicate these disorders are allergy or even immune-mediated in the fish.

Immune reactions to food in companion animals (mostly dogs and cats) involve primarily skin and secondly gastrointestinal tract clinical manifestations (review by Verlinden et al., 2006). Some models of natural sensitization to particular foods (e.g. cow's milk in guinea pigs and wheat gluten in Irish Setter dogs) in companion animals do exist and are particularly relevant to studying similar diseases in humans.

Although an eviction regimen is an important element for the diagnostic of immune reactions to foods, appropriate treatments of food ingredients have been developed for reducing such adverse reactions.

1.9.2.1. Food allergy in farm animals

One major limitation for studying true IgE-mediated food hypersensitivity reactions in veal calves and baby pigs has been the accessibility to anti-bovine IgE and anti-porcine IgE reagents. A murine monoclonal antibody specific for bovine immunoglobulin E was produced in 1988 (Thatcher and Gershwin, 1988) and has been used essentially for studying responses to parasites in bovines. Two papers reported the production of hyper-immune anti-porcine IgE reagents (Roe et al., 1993; Rupa et al., 2008b). Skin passive cutaneous anaphylaxis (PCA) tests were developed as an alternative to IgE assays for studying immune-mediated adverse reactions to food components in young calves.

Milk formula-fed calves

Immune-mediated gastro-intestinal disorders in calves fed milk formulas containing heated soya bean flour were reported for the first time in the early sixties in The Netherlands. The major clinical signs were loss of appetite, diarrhoea and body weight reduction (review by Lalles, 1993). Earlier work in the U.K. based on PCA skin testing concluded to the involvement of either type I (IgE-mediated) or type III (immune-complex mediated) hypersensitivity reactions (Barratt and Porter, 1979; Barratt et al., 1978, 1979; Kilshaw and Sissons, 1979b). The very rapid (< 1 hr) onset of motility disturbances of the small intestine following a challenge with antigenic soya bean in calves already sensitized to this food was also consistent with an IgE-mediated reaction (Lalles et al., 1995a; Lalles et al., 1998; Sissons et al., 1982). Disruption of myoelectric complexes migrating along the small intestine occurred for incorporation rates of soya bean representing 17.5% or more of the total protein in the diet (Lalles et al., 1995a). Histamine via H-1 receptors appeared to be the main mediator involved in motility disorders (Lalles et al., 1995a).

Sensitive calves developed very high levels of circulating IgG_1 antibodies against the major soya bean proteins, namely glycinin and β-conglycinin. Detailed investigations with a set of purified soya bean proteins led to the conclusion that glycinin and β-conglycinin were probably the major adverse food molecules involved but that other minor proteins, including α-conglycinin, Bowman-Birk inhibitor and lectin also induced skin reactions (Lalles et al., 1996b). β-Conglycinin, but not glycinin was also able to induced *in vitro* proliferation of lymphocytes collected from sensitized calves. This, together with long-lasting skin reactions after intradermal injection of β-conglycinin suggested this protein to be involved in type IV, cellular-based hypersentivity reactions in calves (Lalles et al., 1996b).

Histology revealed strong intestinal villous atrophy (Barratt et al., 1978, 1979; Kilshaw and Sissons, 1979a; Lalles et al., 1995a; Seegraber and Morrill, 1982, 1986) associated with increased permeability and decreased absorptive capacity (Kilshaw and Slade, 1980; Lalles et al., 1995a; Seegraber and Morrill, 1979). Increased mast cell numbers and eosinophilia in sensitive calves were reported earlier (review by Lalles et al., 1993). Infiltration of mucosal tissues with T lymphocyte subsets [CD4+ and WC1 (γδ) + T cells in the lamina propria and CD8+ and WC1 (γδ) + T cells in the epithelium] was reported later (Lalles et al., 1996a).

β-Conglycinin was shown to resist gastric digestion *in vivo* (Lalles et al., 1999) and both glycinin and β-conglycinin were detected in small intestinal digesta (Sissons and Thurston, 1984). Treating soya

bean products appropriately (e.g. with heat, proteases or hot water-ethanol mixture) reduced considerably clinical signs of food-related disturbances and improved growth performance (Lalles et al., 1995b; Sissons et al., 1979). The immunogenicity of soya bean products, their digestibility and calf performance could be predicted from the levels of immunoreactive glycinin and β-conglycinin in soya bean products as determined by ELISA (Lalles et al., 1996c; Sissons et al., 1982).
Beside soya bean, veal calves were shown to digest raw field pea flour poorly and to develop severe immune-mediated gastro-intestinal disorders and diarrhoea (Bush et al., 1992). By contrast, fish protein sources (Guilloteau et al., 1986) as well as wheat gluten and potato protein (Branco-Pardal et al., 1995) are well digested and tolerated by young calves.

Baby pigs
Allergy to food (e.g. soya bean) components is suspected to be an aetiological factor involved in the post-weaning diarrhoea syndrome in young pigs (reviews by Dreau and Lalles, 1999; Lalles and Salmon, 1994; Lalles et al., 1993). However, food-specific IgE responses have been studied in piglets only recently (Fu et al., 2007) because of the unavailability of anti-porcine IgE reagents before. Responses to food components were mostly studied regarding the other Ig isotypes and systemic antibodies to food were consistently observed in studies with pigs fed antigenic soya bean products after weaning (Dreau et al., 1994; Friesen et al., 1993; Li et al., 1990). Specific IgM and IgG were found in plasma, and IgM, IgA and IgG in intestinal secretions, in piglets previously fed antigenic proteins (Dreau et al., 1994; review by Lalles and Salmon, 1994). The numbers of plasma cells in the lamina propria of the small intestine were drastically increased following antigenic soya bean consumption (Dreau et al., 1995). Attempts to identify immune mechanisms by direct skin testing suggested that glycinin, by not α- or β-conglycinin were involved in immediate reactions (Dreau et al., 1994). This is consistent with the IgE-mediated reactions to glycinin demonstrated recently (Sun et al., 2008). Gastric administration of glycinin was able to induce dose-dependently an IgE-mediated response in pigs, resulting in diarrhoea and reduced growth performance (Sun et al., 2008). This response was of the Th2 type and was associated with increased numbers of intestinal mast cells, increased histamine release and increased plasma concentrations of IL-4 and IL-10 (Sun et al., 2008). Another recent study identified antigenic (but not allergenic) epitopes of the other soya bean storage globulin, β-conglycinin recognised by pig plasma IgG (Fu et al., 2007). Finally, other proteins from legume grains are immunogenic in pigs but adverse food-related reactions have not been reported against these legumes (Salgado et al., 2002).
Regarding cellular aspects of immune responses to food, lympho-proliferation tests carried out *in vitro* with circulating and intestinal cells added with soya bean extracts or purified proteins remained largely unsuccessful (Dreau et al., 1995; Li et al., 1990; Li et al., 1991). However, local histological investigations demonstrated an infiltration of the intestinal mucosa by T cells of the CD4+ (lamina propria) and CD8+ (epithelium) phenotypes (Dreau et al., 1995).
The neonatal piglet also served to develop models of gastrointestinal allergy (e.g. to peanut and ovomuccoid) (Helm and Burks, 2002; Helm et al., 2003; Helm et al., 2002; Rupa et al., 2008a).

Fish
Adverse reactions to soya bean proteins are suspected to cause deleterious effects on the distal intestine of rainbow trout and Atlantic salmon. However, little evidence is available to date for concluding to particular immune-mediated specific mechanisms. Rumsey et al. (1994) reported increased leukocyte cell numbers and concentrations of plasma protein and immunoglobulin and increased non-specific immunity (activities of neutrophils, monocytes and macrophages) in rainbow trouts fed soya bean products, 'possibly indicating an inflammatory or hypersensitivity response'. In another study, high dietary levels of soya bean were shown to suppress non-specific immunity and to favour the appearance of alterations in distal intestinal tissues in salmon (Burrells et al., 1999). Two studies, one in rainbow trout (Kaushik et al., 1995) and one in salmon (Burrells et al., 1999) reported that no circulating antibody responses to dietary soybean proteins could be found. A Norvegian group noted increased levels of IgM (Bakke-McKellep et al., 2000) and T-cell-like responses to soya bean (Bakke-McKellep et al., 2007) in intestinal tissues of salmon. One possible aetiological factor may be

the presence of lectin that binds intestinal epithelium and cause its disruption in rainbow trout (Buttle et al., 2001).

Horses and birds

Fadok (1995) reported the possibility for pruritic dermatoses 'caused by allergies, including food allergy/intolerance'. However, the lack of recent publications in this area suggests a low incidence of this disease in horses. To the best of our knowledge, immune-mediated reactions to food in poultry have not been reported in the literature thus far.

1.9.2.2. Food allergy in companion animals

Food allergy in dogs and cats is difficult to diagnose accurately because levels of total and specific IgE in plasma are of low predicting value in these animals (review by Day, 2005). Also, as far as dermatological manifestations are concerned, cross clinical signs with responses to frequently carried parasites are common (review by Verlinden et al., 2006). A food trial comprising eviction and reintroduction of the incriminated food/s is probably the best diagnostic tool (Verlinden et al., 2006).

Dogs

Clinical manifestations of food allergy in dogs are mostly dermal, with less than 10-15% gastrointestinal non-specific signs (vomiting, diarrhoea) but other manifestations (e.g. rhinitis, conjunctivitis) can be seen occasionally. Food allergy with dermal manifestation may represent approximately 10 % of all skin diseases, excluding those caused by parasites (review by Verlinden et al., 2006). It is often considered that gender, age, season and breed do not influence the incidence of food allergy in dogs. However, various pure breeds (e.g. Boxer, Collie, German Shepard, Irish Setter) may have a higher risk, contrary to crossbreeds that appear less sensitive. The age of first occurrence of food allergy may vary widely (a few months to > 10 years) but it is generally comprised between 1-2 years. Adverse reactions may happen after 1-2 years of consumption of the incriminated allergens. The most frequent clinical sign of food allergy in dogs is pruritus, although it can resemble other skin diseases (e.g. pyoderma). Otitis interna is a good indication of food allergy in dogs. Diet exclusion is considered the best option for recovery from food allergy. However, the duration of the exclusion treatment can vary widely (from 3 to 13 weeks). Thus, well nutritionally balanced diets are needed for long periods of incriminated food eviction in order to cover the nutritional requirements of the dogs (review by Verlinden et al., 2006).

The food ingredients most frequently incriminated in food sensitivity in dogs are beef and dairy products, followed by cereals, eggs and chicken (Day, 2005; Verlinden et al., 2006). Also reactions to processed (canned or dry) foods are increasingly observed, in connection with the increased consumption of such preparations. Bovine serum albumin, IgG heavy chain and the enzyme phospho-glucomutase were recently identified as major meat allergens in dogs (Martin et al., 2004; Ohmori et al., 2007). Food ingredient (e.g. soya bean protein) hydrolysis is a promising way of preparing hypo-reactive foods for dogs (Biourge et al., 2004; Cave, 2006; Cave and Guilford, 2004; Jackson et al., 2003; Serra et al., 2006).

The Irish Setter dog is a natural model of gluten-sensitive enteropathy occurring at 4-7 months of age and being caused by a type IV cell-mediated hypersensitivity to wheat gluten (review by Verlinden et al., 2006). It is genetically linked to a single major autosomal recessive locus (Batt et al., 1984; Garden et al., 2000; Hall et al., 1992). Various HLA-DQ haplotypes of the canine major histocompatibility complex class II were characterised, one being absent from healthy dogs (Polvi et al., 1997). This disease is characterised by partial to total jejunal villous atrophy associated with a selective reduction in brush border enzyme activities (e.g. alkaline phosphatase, aminopeptidase N and dipeptidyl-peptidase IV; disaccharidase activities are unchanged) and changes in the activity of various intra-cellular enzymes (Batt et al., 1984; Pemberton et al., 1997). Abnormal intestinal permeability is frequently recorded and often precedes the onset of gluten-sensitive enteropathy, suggesting its aetiological role in the disease (Hall and Batt, 1991). Affected dogs display elevated serum levels of IgA, low anti-gliadin antibodies that correlate with immune complex formation, and increased

intestinal mucosa densities of lymphocytes (Hall et al., 1992). Disease relapse can be obtained following a gluten-free diet or a diet with hydrolysed gluten (Hall and Batt, 1992).
Rare diseases, including protein-loosing enteropathy and protein-loosing nephropathy have been described in Soft Coated Wheaten Terrier dogs. They may involve immune complex-mediated, type III hypersensitivity reactions to food (review by Verlinden et al., 2006). Finally, experimentally-induced food allergy models have been developed in dogs (review by Day, 2005).

Cats
Food allergy is rare in cats and when it happens it displays skin manifestations mostly, with less than 10-15% of gastrointestinal disorders (Guilford et al., 2001). The influence of gender, age or breed appears low but some breeds (e.g. Birman and Siamese cats) may be more sensitive (review by Verlinden et al., 2006). The age of first occurrence of allergy and the duration of consumption of the incriminated allergens before the onset of the disease are within the same ranges as in dogs.
The major clinical sign of food allergy in cats is also pruritus showing regional distribution (often head, neck and ears) and being associated with eosinophilia in 20-50% of the cases. Many other clinical signs have been observed at a low frequency (review by Verlinden et al., 2006).
The food ingredients the most frequently incriminated in dietary sensitivity in cats are beef and dairy products, fish and processed (especially dry) foods. Adverse reactions to food additives and to unspecified food components are also relatively high.

Guinea pigs, rats and mice
The young guinea pig is a well known model of natural allergy with high levels of reaginic antibodies and altered intestinal permeability following oral consumption of cow's milk (Heyman et al., 1990; Suzuki et al., 1987). Many other rodent, non-natural (induced) models of allergy have been developed for research purpose (reviews by Fritsche, 2003; Helm and Burks, 2002).

To sum up, immune-mediated reactions have been detected in young farm animals (calves, pigs) and in intensively reared fish species (salmon, trout) in the context of replacing expensive animal proteins (from cow's milk or fish) with cheaper vegetal protein sources, mainly soya bean in food formulas. Food ingredient composition for pets is still diversified which probably explains the corresponding diversity in reported offending foods. Treatment of such food-mediated diseases in animals can be overcome by using food ingredients appropriately treated with single or combined treatments, including heat, enzymes or organic solvents. Interestingly, some immune reactions to foods develop naturally in some animal species (e.g. cow's milk allergy in the guinea pig; gluten sensitive enteropathy in the Irish Setter dog). These have been used as highly relevant experimental models for humans. Finally, additional work is still needed in fish and pets for understanding better the underlying immune mechanisms involved in adverse reactions to food.

1.9.3. Post Market Monitoring of Allergenicity of GM foods

Novel foods in general and food derived from GMOs in particular are submitted to a comprehensive risk assessment before they are approved and launched on the market place. However reactions of consumers may be highly variable, depending on genetic diversity, exposure, geographic, socioeconomic and environmental conditions and all possible situations are difficult to mimic in pre-market risk assessments. In addition some (small) segments of the population who may be very high consumers or particularly susceptible to develop adverse effects when exposed to a novel/GM food may not have been sufficiently accounted for during pre-market assessments. For these reasons the use of post market monitoring programmes (PMM) that aim to provide accurate information regarding actual intake of the novel food by different groups of consumers and occurrence of any expected or unexpected (adverse) effects in relation with the consumption of the novel foods in every day life conditions has long been proposed.

Wal et al. (2003) and Hepburn et al. (2008) noted that large enough study populations should be used to ensure a statistically valid interpretation but emphasized that allergenicity could be a relevant

hypothesis driven case for PMM because allergic individuals constitute targeted groups of the population that may be well identified, organised and informed and who can be motivated to participate actively in a PMM study.

However many difficulties have been identified with regards PMM of foods and particularly of foods derived from GM plants (commodities) as compared to post launch monitoring programmes that are currently performed for medicinal products. Among others this particularly pertains to the need for adequate traceability and identification of the food products derived from the GM source which may be impossible if the GM ingredient is incorporated in a wide range of foods.
This is why the EFSA guidance document recommends the implementation of such PMM only when the GM food has no traditional comparator and is intended for an improved nutritional and health value.

Nevertheless, there is a need to clearly inform medical doctors and consumers of the presence of GM- and non-GM- products, so that case reports can be specifically related to either category of food that has been consumed. In order to have a reliable information between the different stakeholders (e.g. consumers, health professionals, food industry, risk assessors and risk managers), it is crucial that the collection, validation and recording of case reports is carefully checked for relevance (correct clinical diagnosis vs self reported reaction, link to food consumed). Models of such registries of allergic reaction to foods and allergo–vigilance systems have already been developed in some countries and they will certainly be of a great help for risk assessment and risk management. With regards to the possible effects of long term exposure due to the development of cultivation and consumption of new food products on the frequency/severity of allergic sensitization and reaction, more data should be collected to precisely define what the reference "base-line" actually is.

1.10. Conclusions and recommendations

Food allergy is an adverse immune response where sensitization can occur either by cross-reactivity or primary sensitization through the gut. While this is generally assumed to be associated with the production of IgE antibodies to the relevant food other immune mechanisms may be involved. Epidemiological studies have highlighted a food allergy prevalence of 5 to 8 % in infants and young children and less than 5 % in adults. The diagnosis of food allergy is largely based on the clinical history. In suspected IgE-mediated food allergy, the diagnosis will rely on positive skin prick testing by commercial food extracts or native food extracts and serum specific IgE can also be measured to most potential food allergens. Interpretation of positive tests can be difficult as a positive test can also indicate presence of a sensitization without clinical relevance. In addition, food allergy in farm animals has been reported. Some models of natural sensitization to particular foods in companion animals do exist and are particularly relevant to studying similar diseases in humans. Furthermore, adjuvants are substances that may increase the immunogenicity of a protein. An increased IgE response effect is strongly linked to allergy development and should be considered an adverse effect, whereas an IgA reponse is beneficial and, at least, not adverse. Finally, there is a need to inform medical doctors and consumers of the presence of GM- and non-GM- products, so that case reports can be specifically related to either category of food that has been consumed.

1.10.1. Specific assessment in infants and individuals with altered digestive functions

Conclusions

The specific risk of potential allergenicity of GM products in infants as well as individuals with impaired digestive functions (e.g. elderlies, or individuals on antacid medications) should be considered, taking into account the different digestive physiology and sensitivity towards allergens in this subpopulation. Whilst young children may be prone to sensitization with GM derived allergens, this cannot be verified experimentally and clinically due to ethical issues, for which reason the further development of relevant appropriate animal models to provide information on the issue of primary sensitization is recommended. The recommendation provided here should not exclude any age group

or patients on medications affecting the protein digestion, but include these individuals to provide safe GM products to all.

Recommendations

The *in vitro* methods for measuring pepsin resistance of newly expressed proteins should take into account the conditions of individuals with a modified digestion, *e.g.* sub-optimal, higher pH. Besides the oral route of sensitization, also other routes may be considered such as sensitization by aerosolized foods or foods in contact with the skin.

1.10.2. Non-IgE-mediated food allergy

Conclusions

Non-IgE-mediated food allergy is in many ways different with regard to mechanisms from IgE-mediated food allergy, although several syndromes might derive from dual immune reactions, i.e. including IgE-type antibodies as well as other mechanisms (e.g. in eosinophilic diseases).

Recommendations

In the frame of the allergy risk assessement of GM products there is a need to include known data regarding molecular structures of proteins known to be responsible for IgE-mediated allergy but also for other types of immuno-mediated or immuno-toxic reactions such as peptide fragments of gliadin involved in celiac disease.

1.10.3. GM products specifically designed to be either hypoallergenic, adjuvant or vaccinating

Conclusions

For GM products that have specifically been designed to be either hypoallergenic, adjuvant, or vaccinating, such as by gene-silencing of the expression of allergenic proteins or by expression of a antigenic protein fused to an adjuvant protein, clinical studies will probably have to be performed in order to verify the hypothesis of hypoallergenicity.

The outcomes of such clinical studies, including those collected for therapeutic/diagnostic purposes, may also be useful for risk assessment. The design of such studies should therefore also comprise the collection of data useful for risk assessment, such as the inclusion of dose ranges.

1.10.4. Food allergy in food-producing and companion animals

Conclusions

As the pre-market assessment of GM products under Regulation (EC) 1829/2003 also considers the use of a GM product as animal feed besides food, the potential for allergenicity in animals, both companion and livestock, should also be considered. Whilst animals and humans may share some allergens in common, no public source is known to exist to date that provides comprehensive information on specific compounds that would be allergic to some animals but not to humans. In addition, allergy and intolerance to foods in animals may be due to several mechanisms and result in various clinical manifestations. The pathophysiology, the nature of the most common allergens, the level of exposure and digestive physiology in animals may be different from those in humans. Positive indications of a newly expressed protein being similar to an animal allergen may be followed-up with further testing in the pertinent animal, which has to be decided for on a case-by-case basis considering the feasibility of such trials.

Recommendations

It is recommended that in parallel with the human allergen databases, databases for animals are developed and that the level of allergenicity shared between humans and animals is further investigated.

In relation to allergy in animals, livestock animals may serve as "sentinel" given their large and less variable intake of certain feeds, including those derived from GM plants. This might also provide information for allergy in humans.

1.10.5. Adjuvanticity

Conclusions

Adjuvanticity has not been routinely considered in the assessment of potential allergenicity of GMOs. One approach would be to investigate whether adjuvanticity is a problem preferentially in two situations. When a food is known to contain a substance known to functionally or structurally resemble a known strong adjuvant, the possibility of adverse immune responses being caused by the adjuvant should be considered. Further, in cases when adverse immune responses actually are found to be triggered by a product, the presence of strong adjuvants in the product should be considered.

There is no definite test for adjuvanticity, and species differences in adjuvanticity are known to exist. However, because the substance properties and the mechanisms causing adjuvant activity are not well known, experimental work to reveal adjuvant activity of a substance must consist of immune function studies in the intact host. Immunization experiments in rodents with subcutaneous injection may serve for screening and hazard identification (Lovik et al., 2007). However, considering the presumably most relevant route of exposure, peroral immunization models in mice have also been developed at the experimental level (see section on *in vivo* models; (Brunner et al., 2009; Li et al., 2000; Untersmayr et al., 2003; Vinje et al., 2009). An increased IgE response effect is strongly linked to allergy development and should be considered an adverse effect, whereas an IgA reponse is beneficial and, at least, not adverse. With regard to IgM and IgG, it is still very speculative whether an increased response to food allergens may cause adverse effects. Considering the fact that no condition is known in which IgG and IgM antibodies are the primary cause of adverse effects in the intestines, an IgG or IgM adjuvant effect not accompanied by increased IgE production probably should not be considered an adverse effect. However, further research addressing this issue should be undertaken. It is likely that in a near future adjuvant activity of newly expressed proteins or any product derived from GMOs can be assessed using *in vitro* test such as cultures of APC in which a few of the multiple parameters that characterize activation, both at genomic and phenotypic levels can be determined.

To date, such tests may not detect all mechanisms for adjuvanticity/ types of adjuvants nor allow to distinguish IgE and cytotoxic adjuvanticity from IgA/IgG/IgM but they would provide useful information particularly when used in association with animal models (see Annex 6.2.4; 6.3).

1.10.6. Post-market monitoring and prevalence studies

Conclusions

The possibilities for setting up post-market monitoring (PMM) programmes for potential allergies towards GM products should be further explored and particularly the means to clearly identify GM from non-GM products in order to collect accurate data on the actual exposure and significantly and specifically relate any adverse effect to the intake of the GM food in order to substantiate or rule out potential differences of allergies to GM- versus non-GM- products.

There is a need for exchanges of clear and reliable information between stakeholders with regards to the introduction of new food products derived from GMOs, their identification and the adverse effects that their consumption might have caused in consumers. This involves consumers, health professionals, food industry, risk assessors and risk managers. Networks of these groups such as professional and patients' organizations might serve as primary contact point for dissemination and validation of pertinent information.

Occupational allergies of workers exposed repeatedly to relatively high levels of a particular GM product should serve as a sentinel, such as for respiratory allergens, whilst not all allergies caused upon different routes of exposure might be taken into account.

Recommendations

Clear information needs to be provided to the consumers and health professionals on the GM foods available on the market.

PMM should only be recommended on a case-by case basis, e.g. for GM food/feed with altered nutritional composition and modified nutritional value and/or modified to achieve specific health benefits.

Recommendations for futher developement

It is recommended that in case PMM is needed, (a) protocol(s) is/are developed to provide data on the actual intake of the GM food, to guarantee the relevance of the reported adverse effects and to allow establishing any relation/causalities with the consumption of GM foods. It could for instance be valuable to develop prevalence studies in which GM foods are included as potential allergens in order to have a quantitative estimation of the potential impact of cultivation and consumption of GM crops on the prevalence of allergies.

Finally, a more general recommendation which does not only apply for GMOs but for all food allergens is that further research is needed to determine thresholds for sensitization in man and thresholds for elicitation particularly in the case of cross-reactive allergies. This would allow identifying whether there is a level of expression of an allergen in a (GM) food that could be considered “safe”.

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ANNEX 2. STRUCTURAL ASPECTS OF FOOD ALLERGENS: CONFORMATION, IN PLANTA PROCESSING AND FOOD MATRIX INTERACTIONS

2.1. Introduction

One of the key questions that remains to be answered is why some food proteins, and not others, become allergens. Whilst the immune status and genetics of an individual play a role in this, indications are that structural features and biological properties of some proteins predispose them to becoming allergens. Structure-function studies are a major aspect of protein science because they can provide a theoretical framework within which effective predictions can be made. Such relationships and knowledge underlie the *in silico* bioinformatic approaches for predicting the allergenic potential of newly expressed proteins in GMOs as described in Annex 3. Our knowledge base is currently incomplete with regards predicting whether a protein is likely to become allergen through sensitization, an aspect of research hampered by our lack of effective animal models for food allergy (Annex 6). However, our knowledge about the basis of IgE cross-reactivity between known allergens is much greater and hence similarities in structure (both at the sequence level and three-dimensional structures) can be used to predict such cross-reactive allergenicity of newly expressed proteins with much greater assurance. This Annex summarises current understanding of how allergenic potential may be affected by protein structure, biological properties and post-translational processing. This has been placed within the context of how genetics, environmental factors and post-harvest processing may affect expression of allergens in plant foods, and how genetic modification has been used to down-regulate allergen gene expression in food plants to produce low allergen alternatives (see 2.7.3). Similarly our knowledge of how food processing and the food matrix modulate allergenic potential has been summarized. This Annex concludes by identifying gaps in our current knowledge and making recommendations on how information on the structure and biological properties of a novel protein can be used in an integrative fashion to support an assessment as to its likely allergenic potential.

2.2. Classification of allergens based on their structural attributes

The advent of gene cloning led to an explosion in the availability of proteins sequences which allowed more molecular approaches to classifying proteins based on sequence similarities. At the same time there has been an increase in the number of proteins with a defined three-dimensional structure, which has allowed even those proteins with poor sequence similarities to be compared, allowing identification of proteins with low homology levels but superimposable three-dimensional structures to be identified, such as has been the case for the 11S and 7S seed storage globulins (Lawrence et al., 1994). Using this knowledge, and driven by the need to ascribe possible functions to proteins identified simply on the bases of sequenced genomes, has led to the development of several bioinformatic approaches to classifying proteins into families such as the "Pfam" database. This is a large collection of protein sequences (version 23.0 comprises around 10,340 families) which have been classified into protein families using *multiple sequence alignments* and *hidden Markov models"* (http://pfam.sanger.ac.uk/; for further information see Annex 3, (Finn et al., 2006)). It is distinct from the allergen sequence databases discussed in Annex 3. In parallel with such developments there has been an explosion in the identification and sequencing of many allergens. The number of different allergen sequences for is approaching 200 for food alone. Such a data set makes the classification of allergens based on the structural attributes and biological properties feasible (Mills et al., 2004). Previous studies (Aalberse, 2000) have indicated that there is no clear relationship between specific types of tertiary structure element and allergenicity but since the properties of proteins are generally conferred by virtue of their overall three-dimensional structure protein family analysis may pave the way to answering the question what makes one protein, and not another, an allergen.

Protein family analysis of known plant food allergens indicates that the majority fall within just four families accounting for over 65% of the sequences (Jenkins et al., 2005). This pattern has been found

to hold for animal food allergens (Jenkins et al., 2007) and pollen allergens (Radauer and Breiteneder, 2006). The dominant plant food allergen family is the prolamin superfamily, which comprises the cereal seed storage proteins, 2S seed storage albumins, cereal inhibitors of trypsin and α-amylase and the non-specific lipid transfer proteins (nsLTP). This is followed by the cupin superfamily, primarily comprising the 7S and 11S seed storage globulins, the Bet v 1 superfamily, comprising homologues of the major birch pollen allergen, Bet v 1 and profilins. There is a long "tail" in the classification which includes other important allergens such as the class I chitinases involved in the fruit-latex cross-reactive allergy syndrome and the cysteine protease family which includes the kiwi fruit allergen actinidin and the soybean allergen Gly m Bd 30K. It is interesting to note that all these proteins have either seed storage or protective functions. Despite the widespread consumption of vegetative tissues by humans there is little evidence that either the major photosynthetic enzymes or the less abundant structural and metabolic proteins are important allergens, see appendix.

Fewer food allergens of animal origin have been identified to date, reflecting the fact that humans consume a less diverse range of animal-derived foods. Nevertheless these allergens also fall into a relatively small number of structurally-related families. The majority of animal food allergens can also be classified as belonging to one of three families, tropomyosins, EF-hand proteins (with a specific helix-loop-helix motif, which is also present in an important group of pollen allergens) and caseins, with a tail of 14 families containing only one to three reported allergens each. It is of note that several of these "minor" families include allergen families that have been identified as being important inhalant allergens, such as the lipocalin allergens (Virtanen, 2001).

Such a structural classification of allergens is distinct from other classifications which can be based on assessments of allergenic potency. Thus, the Official IUIS Allergen Nomenclature database employs the terms "major" and "minor" for allergens depending on whether more or less than 50% of patients tested react with the corresponding allergen-specific IgE in the given test-system (Larsen and Lowenstein, 1996). This definition is also useful since allergic individuals rarely display a reaction towards only one protein type. However, inevitable such a functional definition is dependent on the panel of patients being studied and hence the definition of major and minor allergens is likely to vary between different allergic patients with different allergic phenotypes or geographic origin (for further details see below).

2.3. Structure-function relationships of allergens

Using structural relatedness expressed in protein family membership, proteins can also be classified on the basis of putative function. This is because common properties of proteins are usually conferred by common structures. One large group are the structural, metabolic, proteins, of which many are regarded as "housekeeping" proteins being essential for the structure and function of all cells. This group includes enzymes involved in biosynthesis and catabolism, structural proteins present in membranes and (for plants) cell walls, transporters and components of signaling cascades. Others may have a protective function whilst in plants there is a large group of storage-related proteins which are especially relevant when considering food as they contribute significantly to the human diet through the consumption of various seeds, nuts and grains.

Several properties conferred by these common structural features have been proposed (Breiteneder and Mills, 2005). However, membership of one of a limited number of protein families in itself is not sufficient to determine allergenic activity. It seems that at least four factors work together to result in the sensitization of an atopic individual with any given allergen, such as (i) the genetic make-up of the exposed person, (ii) the abundancy of the allergen, (iii) the structure of the allergen, and (iv) the biochemical and physicochemical properties of the allergen. This section seeks to summarise observations and current knowledge regarding the latter two points.

2.3.1. Stability

One definition of stability is the ability of a protein to either retain or regain its original native three dimensional structure following treatments (chemical, such as urea, or physical, such as temperature) or to resist attack by proteases. No single type of structure is associated with stability but one structural feature that clearly contributes is disulfide bonds, with both intra- and inter-chain disulfide bonds constraining the three-dimensional scaffold. These covalent links limit the extent to which the protein structure can be disrupted by heat or chaotropic agents and assist the protein to regain its original folded structure once the peturbation is removed. Some notable food allergens are highly disulfide bonded, including members of the prolamin superfamily (nsLTP, 2S albumin, inhibitors of trypsin and α-amylase found in cereals), together with the thaumatin-like proteins (TLPs). However, the absence of disulfide bonds does not indicate a lack of stability since the cupin barrel, for example, is highly stable and possesses no disulfide bonds. Stability also affects the extent to which proteins are attacked by proteases. For example, aspartyl proteases such as pepsin, require a certain degree of flexibility in their substrates since they act on six to eight residue stretches of polypeptide chain which must lie across their active site in an extended conformation. Thus, structural features that increase protein stability, such as extensive disulfide bonding and compactness with few mobile loops, will also render them poor substrates for proteases such as pepsin. However, not all proteins have well-defined three-dimensional structures, and it is now evident that many proteins contain large domains or regions of disordered structure (Dunker et al., 2001). Such proteins are dynamic, with polypeptide chains adopting an ensemble of secondary structures which are in equilibrium with each other. They therefore structurally resemble unfolded, denatured, or partially-folded proteins and have been termed rheomorphic (Holt and Sawyer, 1993). As a consequence of their dynamic nature, rheomorphic proteins do not undergo a sharp transition from one conformational state to another on heating and hence possess many potential thermo-stable epitopes. However, this same flexibility makes them highly susceptible to proteolytic attack. Caseins are examples of unstructured allergens. A more in-depth summary of the current state-of-the-art regarding the role of susceptibility to digestion in determining the allergenic potential of proteins, including the pepsin resistance test, can be found in Annex 4.

2.3.2. Ligand binding

A number of the allergen families described above are able to bind a variety of ligands, ranging from metal ions (such as the parvalbumins) to lipids (such as the nsLTP). Metal ions often become integrated into the three-dimensional structure of a protein with their loss disrupting protein folding and in some instances even resulting in the formation of a partially-folded intermediate. Ligand binding can have an overall effect of reducing mobility of the polypeptide backbone, increasing both the thermal stability and resistance to proteolysis. This is important as many proteases require flexibility in their substrate proteins. Proteins such as the lipocalins and nsLTP which possess a lipid-binding pocket show increased stability when the pocket is occupied (Creamer, 1995; Douliez et al., 2001) .

2.3.3. Lipid/membrane interactions

Many plant food allergens are also able to associate with cell membranes and other types of lipid structures formed in foods. One commonly observed mode of action of proteins that protect plants against microbial pathogens is the destabilization of bacterial or fungal membranes resulting in leakage. Proteins acting in this way include thionins, thaumatin-like proteins, two types of prolamin superfamily members (2S albumins and nsLTPs) and some defensins (Breiteneder and Mills, 2005). In addition to interactions with membrane lipids, many food proteins can interact with other lipids to form emulsified and other structures. Such interactions may be deliberately introduced during the preparation of foods – for example egg proteins with oil to form emulsified sauces such as mayonnaises. Many allergenic food proteins, including whey proteins and caseins are eaten in such emulsified forms. When proteins adsorb to a lipid layer in an emulsion they unfold, revealing hydrophobic regions of the molecules which favour interactions with lipids. The proteins also aggregate to form a two-dimensional gel-like layer which has the elastic properties necessary to

stabilize oil droplets effectively in an emulsion. While emulsions and other lipid structures are widely used as adjuvants in the raising of antibodies, nothing is known on the effect of such interactions on the allergenic potential of foods but it is clear that any protein presented in this form to the immune systems will be at least partially denatured (Breiteneder and Mills, 2005).

2.3.4. Oligomeric and repetitive structures

There is evidence from studies on recombinant therapeutics that aggregation of proteins can enhance immunogenicity (Chirino et al., 2004), even leading to the breakdown of self-non-self discrimination (Rosenberg, 2006). Furthermore elicitation potential may be affected by formation of oligomers and aggregates by providing multiple IgE epitopes which are more effective at cross-linking surface-bound IgE and hence triggering histamine-release in mast cells. Furthermore it appears that aggregates are able to break tolerance to recombinant self-therapeutic proteins such as IFN-α (Braun et al., 1997). For example, dimerisation of the birch pollen allergen, Bet v 1, only gives a positive skin test in sensitized mice when presented in a dimeric, rather than a monomeric state and dimerisation was found to potentiate Bet v 1 specific IgE production (Scholl et al., 2005). It is also intriguing to note that one major epitope site recognised by parasite-neutralising antibodies in malaria corresponds to a serine-rich repeat sequence region (Fox et al., 2002). Food allergens with repeating structures include the tropomyosin allergens of shellfish and seed storage prolamins, oligomeric cupins and many of these proteins are also able to form aggregates.

Impact of modification on structural features and/or on biochemical, physicochemical properties of proteins on allergenicity can not be approached in a general way but on a case by case basis. As knowledge about individual protein families and identification of allergenic epitopes evolves, a more precise risk assessment can be performed.

2.4. Protein structure and IgE cross-reactivity

The molecular mechanism of IgE cross-reactivity, like that of any other type of immunoglobulin, is based on the physico-chemical interactions between an antibody's binding site and a target molecule. Like any molecular recognition event, antibody-antigen interactions are a dialogue between the antibody's binding site and the region on the antigen to which it binds – the epitope (see Annex 1.2).

Given the knowledge on conformational versus linear epitopes, it is apparent that on the basis of molecular mimicry, allergens belonging to proteins with both conserved three-dimensional structures and homologous sequences, such as the Bet v 1 family and the profilins, will exhibit a high degree of IgE-cross reactivity simply because of the way in which they resemble each other. Thus, primary sensitization to birch pollen Bet v 1 elicits an IgE repertoire which is highly likely to recognise one of the many homologues in fruits and vegetables that resemble Bet v 1 so closely (Jenkins et al., 2005). The Bet v 1 family in particular exhibits extraordinary conservation of both surface residues and main chain conformations. In addition, the presence of conserved domains, such as the hevein domain found in the class I chitinases, is also sufficient for IgE cross-reactivity.
Besides the whole protein also fragments of the proteins might be responsible for cross-reactivity.

2.4.1. Cross-reactive carbohydrate determinants

Carbohydrates represent post-translationally derived epitopes (see also Annex 1.2.). According to the work of Mari (Mari, 2002) IgE-linkage to carbohydrates are mostly prevalent in patients with multiple pollen sensitizations, varying from 31% of the pollen allergic patients to 71% in the case of multiple pollen sensitizations. However, the prevalence of IgE to carbohydrates varies from 16-55% in food allergic patients and from 56-79% among patient allergic to hymenoptera (Fotisch and Vieths, 2001).

Most of the reports on pollen allergen glycosylation focused on the asparagine-linked carbohydrate moieties (N-glycans) and showed that α(1-3) fucose and β(1-2) xylose are the major cross-reactive carbohydrate determinants (CCDs) (Andersson and Lidholm, 2003; Fotisch and Vieths, 2001). These N-glycans may be shared by pollen of taxonomically unrelated species (Iacovacci et al., 2001), by pollens and food proteins (Petersen et al., 1996) as well as by plants and insects (Aalberse et al., 1981; Altmann, 2007). However, they are distinct from N-glycans present on mammalian proteins (Altmann, 2007). In helminth infections these fucosylated determinants are known to induce a strong immune response.

IgE responses directed towards plant N-glycans show high cross-reactivity as mentioned above (Aalberse et al., 2001). However, there is debate in the community as to whether IgE-linkage to CCD's is biologically relevant that is translated into clinical significant allergic symptoms (van der Veen et al., 1997). Plant protein extracts displayed much lower ability to stimulate histamine release when compared to purified allergens without N-glycans (Altmann, 2007; Fotisch et al., 1999). The reasons for the low clinical significance is the absence of divalency of glycans that can trigger crosslinking of IgE receptors, low binding affinity of IgE or the presence of blocking antibodies that downregulate the allergic response. Recent data by Jin et al. favour the theory of blocking antibodies, and thus induce tolerance induction, against ubiquitous N-glycan structures (Jin et al., 2008).

However, in the past individual allergens have been identified where glycan structures were involved in allergenic activity as it was shown in the case of celery allergy (Bublin et al., 2003) and tomato allergy (Westphal et al., 2003). Apart from the N-glycans, less frequently, O-glycans can be present on plant proteins and single β-arabinosyl residues linked to hydroxyproline residues play an important role in IgE binding in Art v 1 from mugwort pollen (Leonard et al., 2005). Whether these O-glycans are important in determining the IgE-reactivity of other plant species remains to be identified.

In contrast, little is known about IgE binding to carbohydrates on proteins of animal origin. However, recently, IgE antibodies directed against galactose-α–1,3-galactose, expressed on a range of non-primate mammals, have been shown to cause severe side effects during cetuximab therapy (Chung et al., 2008). In addition, delayed anaphylaxis, angioedema, or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-alpha.1,3-galactose have also been observed (Commins et al., 2009).

2.5. Allergenicity of microbial and fungal proteins

A wide range of fungal allergens have been identified in basidiomycota as well as from ascomycota. They include many housekeeping proteins such as enolases, HSP 70, ribonucleases, manganese-dependent superoxide dismutase (MNSOD) and serine proteases just to name a few (Simon-Nobbe et al., 2008). In general they cause inhalant allergies but there are several case reports of a cross-reactive allergy to the novel food Quorn which is based on *Fusarium*-derived mycoprotein (Hoff et al., 2003; Katona and Kaminski, 2002; Tee et al., 1993; Van Durme et al., 2003). In general individuals were sensitized to respiratory mould allergens and suffered from, sometimes severe, hypersensitivity reactions on consumption of Quorn. In one instance the allergen responsible was identified as a 60S acidic ribosomal protein P2 from *F. culmourum*, which is highly conserved in a number of fungal species (Hoff et al., 2003). Allergic reactions to ingested fungi (mushrooms) (Ho and Hill, 2006; Ichikawa et al., 2006) and mould consumed in fermented foods (Morisset et al., 2003) have been reported but are not generally well documented and no allergens associated with such reactions have been characterized.

Other proteins of microbial origin that can act as respiratory sensitizers following occupational exposure to dusts are enzymes (such as lipases and proteases) used, for example, in the detergent (Baur, 2005). Severe side effects and production of specific IgE antibodies have also been described following vaccination using diphtheriae and tetanus toxoid (Martin-Munoz et al., 2002; Mayorga et al., 2003). Furthermore certain microbial proteins, such as *Stapyhlococcus aureus* protein A, can act as superantigens, inducing IgE responses observed in atopic dermatitis (where it is associated with a dysfunctional epidermal barrier) and is thought to play a role in chronic nasal diseases with nasal polyps (Bachert et al., 2008).

2.6. Post-translational processing of plant proteins and allergenicity

Many plant proteins are processed after synthesis, by the addition of carbohydrate or other chemical groups or by proteolysis. In many cases this occurs within the endomembrane system of the cell and this is particularly important in relation to plant food allergens as the vast majority of these are "secretory" proteins which pass through the endomembrane system to reach their final destination. These include the major plant food allergen families that have been described: the prolamin superfamily, the cupins, cysteine proteinases, Kunitz inhibitors, chitinases and thaumatin-like proteins. Many of these proteins undergo various types of post-translational processing which may affect their allergenic potential. However, we lack experimental data indicating the extent to which post-translational modification may affect allergenic potential, even in model systems. In GM plants there is the potential for the transgenic protein to undergo post-translational processing, which may be different depending upon the host. This, coupled with the gaps in our knowledge, make it difficult to relate studies of allergenic potential of purified transgenic proteins, to the form in which they are found in the GM plant.

2.6.1. Glycosylation

Of the various modifications carried out by enzymes located in the ER and the Golgi apparatus, glycosylation is probably one of the most complex and most relevant regarding allergenic potential given the ability of carbohydrate moieties to act as cross-reactive epitopes (see above). It may also affect protein stability (Wang et al., 1996; Wormald and Dwek, 1999) and many highly glycosylated proteins appear to be more resistant to proteolysis (Gu et al., 1989). N-glycosylation only occurs on asparagine residues in a specific sequence context, i.e. within the three-amino-acid sequence asparagine-any amino acid-serine or threonine. However, the presence of such a site does not always result in glycosylation. Similarly, the extent of glycosylation may vary between two sites on the same protein, as in the bean 7S storage protein phaseolin (Bollini et al., 1983). Modification of N-linked glycans may occur as the proteins move through the stacks of the Golgi, leading to a greater range of complexity, but this is affected by the protein conformation and will not occur if the glycan is inaccessible to the enzymes. The final "trimming" of glycans to remove one or two terminal N-acetylglucosamine residues, occurs soon after the glycoproteins arrive in the vacuole from the Golgi (Kermode and Bewley, 1999).

A second important modification which may occur within the ER is hydroxylation of proline residues. This is particularly important in proteins destined for secretion into the cell wall where the hydroxyproline residues are O-glycosylated in the Golgi apparatus. Hydroxylation of proline residues depends on their sequence context and is catalysed by a specific prolyl hydroxylase enzyme (Kieliszewski, 2001). These residues, along with serine and threonine residues can undergo O-glycosylation in the Golgi. Hydroxyprolines may be modified by arabinoxylation or galactosylation, depending on their sequence context. Thus, contiguous hydroxproline residues appear to be preferentially arabinosylated while clustered but not contiguous residues are preferentially galactosylated (Kieliszewski, 2001; Shpak et al., 2001). Shimizu et al. (2005) also showed that a single proline residue in the sweet potato storage protein sporamin was hydroxylated and modified with arabinogalactan when expressed in tobacco cells, although there is no evidence that this modification occurs in the sweet potato plant (Matsuoka et al., 1995). Differences in the pattern of glycans attached to hydroxyproline have also been observed when an α-amylase inhibitor from bean was expressed in seeds of transgenic pea (Prescott et al., 2005). In this case the recombinant protein was more immunogenic than the native form when fed to mice. In addition to glycosylation, it has been shown that post-translational phosphorylation increased the IgE-binding capacity of caseins.

2.6.2. Post-translational proteolysis

The major protein modification which occurs in the storage vacuole of seeds and other plant storage organs is proteolytic processing. Where vacuolar transit sequences are present as N- or C-terminal peptides these may be removed by specific proteinases. For example, the sweet potato protein sporamin contains a prosequence of 16 residues which directs the protein into the vacuole where it is cleaved between alanine and serine residues (Matsuoka et al., 1990). Many proteins undergo proteolytic processing inside the vacuole, including the 7S and 11S storage globulins and the 2S storage albumins (see chapters in Shewry and Casey, 1999). These storage protein processing events are catalysed by a specific group of cysteine proteinases, called legumains, which cleave at the C-terminal side of asparagine residues (Muntz, 1998) although other proteases have also been implicated (Gruis et al., 2004). One example of this is the presence of both an unprocessed and a processed 2S albumin isofoms of Ara h 6. The processed isoform has undergone a limited proteolysis with a loss of an internal dipeptide. The impact of the processing on the allergenicity was low since the structure of the protein was maintained by the disulfide bonds (Bernard et al., 2007). In addition, other trimming at the N- and C-termini is responsible for further heterogeneity in this group of proteins (Moreno et al., 2004). Thus, proteolytic processing could result in the exposure of different parts of the protein to those in the intact forms and inauthentic processing could result in the presentation of different epitopes with impacts on allergenicity.

Any mutations which result in failure of the proteins to fold correctly may result in the protein being removed from the endoplasmic reticulum (ER) and degraded (Napier, 1999; Pedrazzini et al., 1997), although accumulation may occur if the protein remains tethered to the ER membrane (Gillikin et al., 1997; Kim et al., 2004). Such proteins may expose different residues to the immune system leading to altered immunogenicity/allergenicity compared with the native, correctly folded protein.

2.7. Whole plant allergenicity

As indicated in Annex 1.2, a hierarchy of different levels of structures running from whole food to protein molecules to epitopes located within proteins has to be taken into consideration when investigating the allergenicity of a food.

This section deals with the allergenicity of the whole plant and derived products and particularly with regards to possible alterations, e.g. due to over-expression of natural endogenous allergens, as an unintended effect of the genetic modification

2.7.1. Natural genetic variation and allergen abundancy

In the recent past the variation in allergenicity in a range of cultivars within one plant species has been characterised. Thus, out of a panel of 18 date cultivars 5 highly allergenic lines were identified by means of SPT and IgE ELISA reactivity (Kwaasi et al., 2000). Bell pepper cultivars (n=8) were analysed for the presence and levels of the IgE binding Bet v 1 homologue, an osmotin-like protein (PR 5 protein), and profilin which showed differential expression patterns (Jensen-Jarolim et al., 1998). Koppelman and colleagues (2001) analysed 13 different peanut samples (4 varieties) derived from different geographical locations. No significant difference could be found between these samples concerning the allergenic activity. Since the 1960s the green kiwi, *A. deliciosa* is on the market in Europe. In the last few years another kiwi species, *Actinidia chinensis cv. Hort 16 ZESPRI* Gold, more commonly known as golden kiwi, has become available. Actinidin, Act c 1, previously identified as the major allergen from the green kiwi, has not been identified as IgE binding component in the golden kiwi (Bublin et al., 2004). In contrast other allergens e.g. the thaumatin-like protein are common in both species. For 10 soybean cultivars clear differences in IgE binding potencies could be identified *in vivo* and *in vitro* (Codina et al., 2003). A total of 88 apple cultivars has been analysed for allergenicity by *in vitro* IgE tests and *in vivo* tests (SPTs, and oral challenges in a smaller number of cultivars) (Sancho et al., 2008). Significant differences in allergen levels regarding Mal d 1 and Mal d 3 translated into IgE dependent reactivities *in vitro* as well as *in vivo* (Bolhaar et al., 2005; Sancho et al., 2008).

However, the IgE reactivitiy to individual allergens can differ between individual patient groups as it has been shown for the apple allergens. IgE reactivity to Mal d 1 is based on previous sensitization to the birch pollen homologue Bet v 1, and is predominant in areas where birch trees are flowering, that are Northern and Central Europe. Usually this pollen related fruit allergy is linked with rather mild allergic symptoms of the oral allergy syndrome as it has been shown in an EC funded project SAFE (Fernandez-Rivas et al., 2006; Hoffmann-Sommergruber, 2005). A different sensitization pattern is observed in apple allergic patients from Southern Europe. These patients display IgE reactivity predominantely to the non-pollen related nsLTP, Mal d 3. In addition, Mal d 3 was identified as a risk factor for developing severe symptoms upon consumption of fruits (Fernandez-Rivas et al., 2006). Similarly different sensitization patterns have been reported for cherry allergens, Pru av 1 and Pru av 3 (Reuter et al., 2006) and for hazelnut allergens (Pastorello et al., 2002). All these studies provide evidence that allergic patients groups differ in their sensitization patterns due to exposure to different inhalant allergens, due to different consumption habits and maybe additional environmental factors. These different sensitization patterns may in turn influence the severity of reported symptoms as it is observed for the pollen-related food allergens versus the non pollen-related food allergens. Therefore, fruit cultivars low in one allergen presenting a benefit of one allergic patients' group may still represent a risk for another different allergic consumer group.

Identification of allergen encoding genes and their genomic mapping provides additional information about the potential allergenicity of a cultivar and or species. So far, gene mapping has only been performed on the apple genome locating the four identified allergens, Mal d 1 (a Bet v 1 homologue), Mal d 2 (a profilin), Mal d 3 (a lipid transfer protein) and Mal d 4 (a thuamatin-like protein). The gene families of the respective allergens were determined (Gao et al., 2005a; Gao et al., 2005b; Gao et al., 2005c) and Mal d 1 related markers were identified which could help in new breeding programs aiming at low allergen variants (Hoffmann-Sommergruber et al., 2007).

2.7.2. Post-harvest treatments and allergen abundancy

In addition to genetic variation, environmental factors also affect plant gene expression including allergens. Thus site-to-site variation can affect levels in expression, with agronomic factors, and climate all playing a part. Furthermore the stage of ripening of fruits, such as apples, may also affect the allergen content in fruits and vegetables. Post-harvest treatment such as storage conditions (temperature, modified atmosphere) of fruits and vegetables can also increase or decrease the allergen load as it has been shown for apples regarding Mal d 1 and Mal d 3 concentrations in relation to normal air versus controlled atmosphere versus upon ambient conditions (Sancho et al., 2006a; Sancho et al., 2006b). For example, within a single apple cultivar the allergen levels can differ from individual fruits up to ten-fold.

In contrast, postharvest ripening treatment on mango did not exert changes in the levels of the 2 known allergens, Man I 1 and Man I 2 (Paschke et al., 2001). These few examples show a broad variation range of allergen levels in raw plant food which makes it difficult to pinpoint general acceptable allergen levels in certain allergenic fruits and vegetables even if the necessary detection assays are available.

Furthermore, in most plant food allergies a multiplicity of allergens has been implicated and hence a decreased concentration of a single protein may not be sufficient to make a food safe of allergic consumers to eat. Therefore, detailed information on the allergenic repertoire of a given food is mandatory, as well as an in-depth characterization of the individual allergens and their performance during storage and food processing. In addition, the allergen recognition pattern may vary among different populations according to their exposure, dietary habits and environmental factors such as pollen exposure.

2.7.3. Transgenic plants downregulating expression of allergens

Genetic modification has also been applied to down-regulate levels of allergens in plant foods with the aim of developing low allergen alternatives. Single-site mutagenesis of two IgE binding peptides of

the soybean allergen, Gly m Bd 30 kDa has been proven to be effective in producing a hypoallergenic soybean protein (Herman et al., 2003). An alternative approach was taken using antisense RNA for the 14 kDa and 16 kDa allergenic proteins in rice, which repressed the allergen gene expression in maturing seeds and resulted in the reduced allergenicity (Nakamura and Matsuda, 1996; Tada et al., 1996). The same method was applied to the soybean, targeting the Gly m Bd 30 kDa gene and after successful transformation, this protein could no longer be detected (Herman et al., 2003). Further examples were shown for downregulating the major apple allergen, Mal d 1 (Gilissen et al., 2005) and apple plantlets were virtually free of Mal d 1 as shown by immunoblots and skin prick tests. Recently transgenic tomato fruits suppressing expression of tomato profilin (Le et al., 2006) and non-specific lipid transfer protein (Lorenz et al., 2006) were obtained by applying the double-stranded RNA interference (dsRNAi) technology. Although these "hypoallergenic" plants represent valuable alternatives for the allergic consumer the acceptance of such beneficial GMOs is rather low as evaluated by a survey performed in 3 European countries (Miles et al., 2006). At present these approaches are of scientific value and highlight the possibility to downregulate individual allergen levels through genetic modification. However, the impact on the allergenicity of the whole food remains to be demonstrated on a case-by-case basis.

2.7.4. Food Processing and the matrix

Food processing can impact on the allergenicity of foods (for review see Mills et al., 2006). For example the removal of tissues containing allergens may reduce allergenicity of foods, as has been found for peaches where removal of the skin which contains the nsLTP allergen, reduced their allergenicity (Brenna et al., 2000). Another example is the leaching of peanut 2S albumins into the cooking water during boiling (Mondoulet et al., 2005). Processing of ingredients and preparation of finished foods may also affect allergenicity by altering the structure and properties of food proteins. Most studies to date have focused on the effect of processing on elicitation of allergic reactions in sensitized individuals and there is little data on effects on sensitization potential. Effects appear to depend on the structural characteristics of the allergen, with highly stable proteins, such as members of the prolamin superfamily such as 2S albumins and nsLTP tending to retain their allergenic properties after severe processing procedures, such as fermentation (Asero et al., 2001) whilst others, notably the Bet v 1 homologues appear to be more labile, frequently loosing their allergenicity in processed foods. Thus, processing of fresh fruits, such as apple, removes the ability of the apple Bet v 1 homologue, to elicit allergic reactions in sensitized individuals (Asero et al., 2006) but for other foods, such as celery root (celeriac), the Bet v 1 homologues (Api g 1 in this instance) retains its eliciting potential after cooking (Ballmer-Weber et al., 2002). Recent studies of Bet v 1 itself show it is relatively thermostable, the protein unfolding only at temperatures above 68°C (Mogensen et al., 2007), implying that differences in the plant tissue matrix of apple compared to celeriac may modulate the stability of this family of proteins to food processing.

The role of the food matrix in determining allergenicity is not generally well understood. It has been shown during double blind placebo controlled food challenge that the form in which a protein is given can affect the development and severity of allergic reactions to peanut (Grimshaw et al., 2003). How the food matrix might impact on the sensitization phase of food allergies is not understood. Studies in this area are in their infancy because of the complexities presented by food structures and components interactions, and the problems presented by a lack of truly effective animal models for food allergy. Indications are that food component interactions may affect the way in which allergens are released from foods, the way in which they are digested and subsequently taken up and interact with the immune system. Certainly it appears the co-adminstration of a mixture of lipids from Brazil nut is essential for the sensitizing potential of the Brazil nut 2S albumin allergen, Ber e 1, in particular animal models (Dearman et al., 2007). It remains to be determined whether this is a synergistic effect of lipids acting on the immune system in conjunction with the protein, and/or effects of lipid structure on release and presentation of the allergen.

Within the food matrix a protein may undergo Maillard modification i.e. non-enzymatic glycosylation of proteins during food processing. Maillard reactions takes place between free amino groups (generally lysine residues) on proteins with reducing sugars (such as glucose and lactose) and the subsequent rearrangements lead to formation of a complex mixture of products. N-glycosylated forms of proteins may be more allergenic than their unmodified counterparts (Davis et al., 2001). The Maillard reaction was shown to play a possible role in the allergenicity of foods such as peanuts (Beyer et al., 2001; Maleki et al., 2000) and appear to enhance the IgE-binding capacity of the shellfish allergen, tropomyosins (Nakamura et al., 2005). However, different allergens appear to respond in different ways. Thus, Maillard reactions significantly reduced IgE reactivity of the allergenic Bet v 1 homologue of cherry, Pru av 1 (Gruber et al., 2004), whilst this reaction was found to protect the IgE-binding capacity of the allergenic nsLTP of apple following harsh thermal treatment (Sancho et al., 2005). Maillard modifications are only one of a range of chemical changes that can take place in foods during cooking.

Another modification catalysed by the polyphenol oxidase, is responsible for enzymatic browning reactions in fresh fruits and vegetables. Modification of Pru av 1 with epichatechin and caffeic acid reduced IgE-binding capacity although the extent to which it was reduced was highly dependent on the polyphenol involved, quercetin and quercetin glycoside, rutin, having a lesser effect (Gruber et al., 2004).

2.8. Conclusions and recommendations

The risk assessment of GMOs is based on a comparative approach with regards to allergenicity. It aims to establish whether the potential allergenicity of the GM crop is less, equal or increased compared to that of the non-GM counterpart (EFSA, 2006). A prerequisite for assessing any potential increase in allergenicity of a GMO should be to define allergen levels in wild-type species and cultivars as a "baseline" reference. Data on quantitative allergen expression levels from either raw or processed food are scarce. Our knowledge on the impact of both natural (plant tissue) and processed structures on allergenic potential (sensitization or elicitation) is poor. Consequently, it is difficult to predict how processing and the matrix may affect allergenic potential. Whilst it is difficult to predict the likelihood of a newly expressed protein in a GMO to sensitize an individual, we can predict, based on sequence and structural similarity the ability of proteins to elicit an allergic reaction in individuals already sensitized, such as in the cross-reactive pollen-fruit/vegetable and latex-fruit allergy syndromes. Based on the existing knowledge about three dimensional properties of known allergenic protein families there is good evidence to predict a potential new allergen if this protein displays one or more of such identified characteristics. Recent findings in the area of protein structure (determination of 3D structures by X-ray crystallography and NMR) coupled with modern computational methods enabling effective molecular modeling have provided a new means of classifying proteins and provide the opportunity to link structure with function (allergenicity). Such knowledge is a prerequisite for prediction of allergenic potential although this can only be fully realized when a potential allergen meets the immune system of an atopic individual. Working towards a better understanding why some proteins and not others can become allergens several aspects need to be addressed and considered.

2.8.1. Endogenous allergenicity and references for the comparative assessment of the whole GMO

Conclusions

The risk assessment of GMOs is based on a comparative approach with regards to allergenicity (EFSA, 2006). This principle applied to allergenic risk assessment of GMOs requires that the allergenic repertoire of the host plant is known, including the natural variation in the levels of expression of allergens in the different (edible) tissues (and pollen) of the plant and the possible mixture of isoforms (if applicable) found in the non-GM isogenic counterpart. The effect of the genetic modification on the expression of the natural endogenous allergens can be set within the context of expression in the unmodified crop. A prerequisite for assessing any potential increase in

allergenicity of a GMO is to define allergen levels in wild-type species and cultivars as a "baseline" reference. Although the number of identified food allergens has increased tremendously in the recent past, little is known about actual allergen concentrations in plant foods and even less is known about the potency of individual allergens with regards threshold levels of allergens set for sensitization and elicitation of allergic reactions by individual foods.

The allergen repertoire of individual plant food species and the variation in levels of allergen expression in edible tissues of plants needs to be defined. This should be linked to indices of potency for different allergen molecules. Integration of such data sets will allow any changes in the allergen repertoire of a GMO to be quantified and linked to potential changes in sensitization/elicitation potential.

Recommendations

In order to assess the "allergenic" potency of a GMO, its endogenous allergen repertoire needs to be compared in a qualitative and, where possible, (semi-) quantitative fashion, with that of the wild-type counterpart; i.e. in a comparative safety assessment.

A comparison of the allergen repertoire of the GM and conventional crop should be performed using individual human allergic sera, if applicable. This is still considered a reference procedure in order that the comparison does not overcome minor allergen(s). However, modern proteomic and mass spectrometry methods, including high throughput analytical techniques of proteins, are also able to provide qualitative and quantitative information on the levels of the different allergens and have the major advantage of not depending on reagents of human origin (see Annex 5).

In the case of recipient plants known to be common allergenic foods, the test, control and reference crops should be grown, samples stored and processed under conditions that are as identical as possible because agronomic and post-harvest treatments are known to have a considerable effect on allergen expression levels. When the allergens of clinical importance are limited, identified and recorded, a thorough comparison between the GM plant and the non-GM appropriate comparator should not be precluded because of natural variability.

Information should be provided on the contents and/or qualitative and quantitative profiles of endogenous allergens in multiple commercial non-GM varieties that are commonly grown for food and feed production and in the GM crop varieties containing the GM event that are to be commercialized. The outcomes of the comparison of GM and non-GM should be interpreted in the light of the natural variability in intrinsic allergenicity and with regards to the strategy used for the genetic modification (e.g. choice of the recipient cultivar for the GM event) on a case-by-case basis. Significant differences should be identified even if they range within the natural variability of commercially available cultivars.

The choice of the reference crops used for establishing the natural range of variability should be limited to the most commonly grown cultivars and thus reflecting the expected range of human and animal exposure.

2.8.2. Structure, biological properties and allergenicity prediction of the newly expressed proteins

Conclusions

The frequency of a protein family in a given genome is not reflected in the frequency distribution of food allergens. The distribution observed for food allergens is similar to that of pollen allergens (Radauer and Breiteneder, 2006); its highly restricted nature is striking and emphasizes the fact that whilst, in theory, all proteins have the potential to become allergens, in practice this is not the case. Membership of a particular protein family is indicative of a protein being more likely to be an

allergen, than a protein which does not belong to a proteins family. It is not predictive *per se* but is related to the properties of proteins which are conferred by their three-dimensional structures.

Whilst it is difficult to predict the likelihood of a novel protein as represented by a newly expressed protein in a GMO to sensitize an individual, we can predict, based on sequence and structural similarity the ability of proteins to elicit an allergic reaction in individuals already sensitized, such as in the cross-reactive pollen-fruit/vegetable and latex-fruit allergy syndromes. Based on the existing knowledge about three dimensional properties of known allergenic protein families there is good evidence to predict a potential new allergen if this protein displays one or more of such identified characteristics. Reversely, if a certain protein does not display already known structures linked with allergenicity it does not mean total absence of potential allergenicity. This applies especially to proteins that human mucosal surfaces have not been exposed to before. In this case allergenic risk assessment may need to be re-evaluated after a few years of consumption of the GMO.

We need to understand why some proteins, and not others, can become allergens. As part of addressing this question we need to understand how routes of exposure, levels of exposure and digestion affect sensitization potential. Thresholds for sensitization and tolerisation need to be identified in man and related to thresholds for elicitation in cross-reactive allergies to identify whether there is a level of expression of an allergen in a food that could be considered "safe". This is especially important for making allergenic risk assessment of proteins expressed in low amounts versus those expressed to a significant proportion of the plant tissue. Studies carried out using purified target proteins prepared by expression in organisms such as *Escherichia coli* need to be related to the properties of the protein as expressed in the plant, thus taking into account post-translational modifications, such as proteolysis and glycosylation, that only occur in the plant. Modern protein mass spectrometry methods can enable such comparisons to be made.

Recommendations

Without a knowledge of the mechanisms whereby one protein, and not another, becomes allergenic, prediction will always be uncertain. However, on a case-by-case basis, information on protein scaffolds found in protein families that contain many non-homologous allergens may be informative when used in combination with other factors such as stability to processing, proteolysis and levels of consumption. They may be especially important in understanding IgE cross-reactivity as it is the conformational relationships which underly and explain much of this phenomenon (Breiteneder and Mills, 2006).

2.8.3. Impact of post-translational processing and expression in the plant of the trait proteins

Conclusions

Evaluating allergenic potential needs to take account of effects of post-translational modifications and relevant processing-induced modifications on either the background allergen repertoire of a food crop or the trait itself. Whilst methods for direct analysis of N-linked carbohydrates on proteins are well developed, those for mapping of relevant O-linked carbohydrates are complex and require large amounts of material and complex chemistry for their analysis; they also show more heterogeneity than N-linked glycans. Furthermore given the difficulty of predicting the impact of a given plant tissue or a different plant species on the post-translational processing of a novel protein, any assessment of allergenic potential needs to be done on the tissues as it would be eaten and not simply on a purified protein. It is clear that plant proteins may undergo highly specific processing (including proteolysis, glycosylation and a number of other post-translational modifications) within the endomembrane system. Furthermore, the pattern of processing is determined by the sequence context of the potential processing sites, the accessibility of the sites to the processing enzymes and the endomembrane compartment in which the protein is retained or passed through. When the test material used for assessment of allergenicity of the newly expressed protein has been produced in a microorganism, it should be verified that its structure is the same as the structure of the protein expressed in the GM plant, including all post-translational modifications. Furthermore, it is not always possible to predict

what modifications will take place and direct analyses are required. Finally, because the endoplasmic reticulum and vacuole provide convenient destinations for the targeting of proteins in transgenic plants these events may occur more frequently with expressed foreign proteins than would be expected based on their overall occurrence *in vivo*. Our knowledge of how post-translational processing may affect allergenic potential is largely confined to effects of glycosylation and influence of cross-reactive carbohydrate determinants.

Improved methods for mapping of O-linked carbohydrates would facilitate more effective analysis of these post-translational modifications.

Recommendations

It is recommended to define the presence and nature of all post-translational modifications of the newly expressed protein in the plant and to compare it to the original native protein by the use of up to date proteomics methods. Mass spectrometry is proven as a powerful method to detect post-translational modifications by shifts in mass data.

Recommendations for further development

A distinction should be made between IgE binding capacity linked with clinical significance versus positive IgE binding without clinical significance (e.g. N-linked glycans).

Data on the level of expression of the newly expressed proteins in edible tissues needs to be established. This should include expression levels in different tissues, and indications of how agronomic conditions, developmental stage and post-harvest storage affect these. These then need to be linked with data on thresholds regarding sensitization and elicitation of allergic reactions in cross-reactive allergy syndromes. This also needs to be linked to an assessment of how genetic modification may affect the levels and expression of allergens in the wild-type crop in order to assess the effect of genetic modification on the intrinsic allergenicity of the crop.

2.8.4. Impact of food processing and the matrix on the allergenicity of newly expressed proteins

Conclusions

Depending on the food or tissue in which a novel protein might be consumed the effect of the food structure on allergen release in the alimentary tract, bioaccessibility, digestion and allergenic potential should be assessed. Our knowledge on the impact of both natural (plant tissue) structures and processed structures on allergenic potential (sensitization or elicitation) is poor. Consequently it is difficult to predict how processing and the matrix may affect allergenic potential.

We need a better understanding of the mechanisms underlying what makes some proteins more allergenic than others and how some food structures (such as lipid-rich foods) may affect release and immunological properties of allergens in foods, as well as their stability to digestion. Such knowledge would allow any risk assessment to be better informed and hence offer greater assurance to consumers. Such knowledge would also help underpin the development of bioinformatic methods with an improved capability of predicting allergenic potential. In addition post-genomic methods need to be developed and applied to mapping processing-induced modifications.

Recommendations for further development

Data on the impact of relevant processing-induced modifications, (such as Maillard modifications) on stability to digestion and allergenic potential of the newly expressed protein need to be considered. This needs to be complemented by data on how food processing and the matrix affect the release of a newly expressed protein from a food derived from GMO and prepared as it is intended to be consumed. Such data needs to be linked to assessing how the digestibility and immune-reactivity is affected.

2.9. Appendix

Plant food allergen families

Prolamin superfamily

First identified on the basis of visual comparison of amino acid sequences, this protein superfamily is characterised by a conserved pattern of cysteine residues, which are located within a sequence of about 100 amino acid. Either six (for the 2S albumins and ns LTPs) or eight (in the trypsin and α-amylase inhibitors) such cysteine residues are present, which form three or four intra-chain disulphide bonds. Most of the proteins contain characteristic Cys Cys and Cys X Cys motifs, where X represents any other residue, and can be defined by the formula:

Cys-(X=7-13)-Cys-(X=8-26)-Cys-Cys- (X=8-30)-Cys-X-Cys-(X=20-48)-Cys-Cys

This "cysteine skeleton" has been disrupted in some cereal seed storage prolamins (notably the high molecular weight subunits of wheat glutenin) by the insertion of a repetitive domain, with the component cysteine residues being present in the N- and C-terminal portions of the proteins. Whilst the degree of sequence identity between the conserved regions of prolamin superfamily members is low they are structurally conserved with very similar three-dimensional structures (Mills et al., 2003).

Whilst the pattern of cysteine residues is conserved, they are connected to form different disulphide bonds in the nsLTP sub-family compared with the 2S albumin of α-amylase inhibitors. This difference reflects the fact that only the nsLTPs possess a central lipid-binding tunnel and is a rare example of the protein sequence being more highly conserved than the 3D protein structure. At present members of the superfamily have only been identified in plants and it has not been possible to identify its evolutionary origin in primitive organisms. Most members function as either seed storage proteins (such as the cereal seed storage prolamins) or in plant protection such as the α-amylase inhibitors and nsLTPs, the latter belonging to pathogenesis-related (PR) protein group 14 (Van Loon and Van Strien, 1999), or a combination of the two (2S albumins). However, they also include a group of structural cell wall proteins (Jose-Estanyol and Puigdomenech, 2000).

The prolamin seed storage proteins also trigger an immune-mediated food intolerance disease known as coeliac disease which is thought to affect around 1% of the population in Western Europe. This has a different immune mechanisms to type I IgE-mediated allergic reactions and symptoms can take much longer (hours, days) to manifest themselves compared to IgE-mediated reactions which usually take place more rapidly (minutes, hours). Coeliac disease results from an abnormal cellular-mediated immune response which causes an inflammatory reaction in the small bowel and results in flattening of the mucosa and an associated malabsorption syndrome (Walkersmith et al., 1990). It is a complex disorder which has many manifestations unrelated to the gastrointestinal tract which include defective tooth enamel and myocardiopathy amongst many others (Hischenhuber et al., 2006). Gluten intolerance is thought to arise as a consequence of deamidation of glutamine residues in peptides resulting from activity of the tissue transglutaminase present in the gut mucosa. The modified peptides are able to bind to class II human histocompatibility leucocyte antigen (HLA) molecules DQ2 and DQ8. This recognition event appears to orchestrate an inflammatory response which results in the flattened mucosa characteristic of coeliac disease (Hischenhuber et al., 2006).

Cupins

The cupins are a superfamily of proteins which possess a common β-barrel structure which are thought to have evolved from a common ancestor on the basis of two shared sequence motifs, [G(X)5HXH(X)11G] and [G(X)5P(X)4H(X)3N], (where X is any amino acid residue) which correspond to a metal binding site in many, but not all, members of the superfamily (Dunwell et al., 2004). The structure is thought to have evolved from an ancestral protein present in prokaryotes through fungi, to flowering and non-flowering plants. Sub-families include the germins and sporulins, which possess only single β-barrel domains with enzymatic activity and a metal ion (manganese) located at the centre of the barrel (Woo et al., 2000). Germins from bell-pepper (Leitner et al., 1998)

and orange (Ahrazem et al., 2006) have been identified as allergens. Another important sub-family are the bicupin seed storage globulins where two β-barrel domains have been fused to form subunits which are then assembled into either trimeric (7S globulins) or hexameric (11S globulins) structures (Mills et al., 2003).

Bet v 1 family

Bet v 1 was one of the first cloned allergens and is the major allergen in birch pollen (Breiteneder et al., 1989) and now 23 homologues with known sequence (http://www.meduniwien.ac.at/allergens/allfam/) in a wide variety of plant species have been characterised indicating the ubiquitous allergenic nature of this protein in the plant kingdom. The Bet v 1 proteins have no clearly ascribed biological function but may be involved in plant protection, belonging to the PR 10 group of pathogenesis related proteins (Van Loon et al., 2006; Van Loon and Van Strien, 1999). A striking sequence motif GXGXXG is present in most Bet v 1 proteins but is reduced in some cases to GXG. This is known as a P-loop (phosphate binding loop) and is frequently found in protein kinases and nucleotide binding proteins (Saraste et al., 1990). However, the nucleotide binding function was determined experimentally for Bet v 1. The protein Bet v 1 possesses a central tunnel which appears to bind plant steroids (Markovic-Housley et al., 2003; Neudecker et al., 2001) but the physiological role of this property has not been established. However, the high degree of homology of Bet v 1 proteins across diverse plant species (Jenkins et al., 2005), which even extends to surface features which are generally highly variable between species, indicates that they do have a conserved function. This high level of structural conservation is also important for their cross-reactions as allergens.

Profilins

Profilins are small (12-15 kD), ubiquitous cytosolic proteins, which are present in all eukaryotic cells and act as actin-binding proteins (Witke, 2004). As such they may play a key role in regulating intracellular transport processes and cell morphogenesis and division. Despite having low sequence similarity these proteins are structurally homologous across lower eukaryotes, plants and animals, with a compact globular structure consisting of a central seven-stranded anti-parallel β-sheet enclosed by the N- and C-terminal α-helices on one side and one or two helices on the other side. The plant homologues are somewhat divergent, notably with a slightly longer solvent exposed loop between the N-terminal α-helix and the first β-strand which is more variable and represents part of an IgE epitope in the allergenic profilin from birch pollen, Bet v 2 (Fedorov et al., 1997). The profilins are also involved in the cross-reactive pollen-fruit allergies. However, the clinical significance of IgE reactivity to either pollen and fruit profilins differs.

Animal allergen families

Fewer animal allergens have been identified to date, which perhaps reflects the fact that humans consume a less diverse range of animal-derived foods. Nevertheless these allergens fall into a relatively small number of structurally-related families (Jenkins et al., 2007).

Tropomyosins

Tropomyosins are highly conserved proteins as a consequence of the key regulatory role they play in muscle contraction together with actin and myosin. Tropomyosins have highly conserved structures, which relates to the regulatory role that they play in muscle contraction, in combination with actin and myosin. They are rod-shaped coiled-coil dimers which form head-to-tail polymers along the length of an actin filament (Phillips et al., 1979). Like profilins, they are present in all eukaryotes but the allergenic tropomyosins are confined to invertebrates (Ayuso et al., 1999), primarily two groups, crustaceans and molluscs, generally referred to as shellfish (Wild and Lehrer, 2005). As a result of sequence similarity between tropomyosins from different species, the IgE from the sera of some allergic individuals who are allergic to crustaceans may also bind to tropomyosins from several molluscan species (Leung et al., 1996) but not to vertebrate tropomyosins (Ayuso et al., 1999). The

introduction of mutations to "humanize" the sequence reduces or abolishes IgE binding (Reese et al., 2005).

Parvalbumins

The parvalbumins are a class of denaturation-resistant calcium-binding proteins that are important for the relaxation of muscle fibers by binding free calcium in cells. They are present in high amounts in white muscle of fish and amphibians, and in lower amounts in fast twitch muscle of birds and mammals. They contain calcium-binding E-F hand motifs (Pauls et al., 1996) that are related to motifs for other allergenic calcium-binding proteins such as polcalcins from pollen (Ledesma et al., 2006) and troponin c from cockroaches (Hindley et al., 2006). Fish β-parvalbumins have been identified as allergens in a large number of fish species and in frog (Hamada et al., 2004; Hilger et al., 2004; Wild and Lehrer, 2005). It is not clear whether fish parvalbumins are the primary sensitizing agent, and that homologues from molluscs and frog are allergenic because of IgE cross-reactivity or whether non-fish parvalbumins are able to sensitize *per se.* It is possible that the cross-reactivity between β–parvalbumins results from the conservation of surface structures, as has been suggested for some plant food allergens (Jenkins et al., 2007; Jenkins et al., 2005).

Caseins

Caseins are exclusively mammalian proteins. Casein, Bos d 8, is actually an association of different proteins that constitute the coagulum, i.e. the solid fraction of proteins obtained after coagulation of milk. Each individual casein, α_{S1}-, β-, α_{S2}- and κ-casein, represents a well-defined chemical compound but they cross-link to form ordered aggregates: micelles. They are structurally mobile proteins. α_{s1} - , α_{s2}- and β-caseins have a dipolar-type structure, comprising a globular hydrophobic domain and a highly solvated and charged domain, with amphipatic properties and bind calcium through clusters of phosphoserine residues. The caseins form a shell around amorphous calcium phosphate to form microstructures called nanoclusters allowing calcium levels in milk to exceed the solubility limit of calcium phosphate. These nanoclusters are assembled into the casein micelles found in milk, which are in turn stabilized by κ-casein (Tuinier and de Kruif, 2002). Because of their structure caseins are very susceptible to all proteinases and exopeptidases but not significantly affected by severe heat treatments. The heterogeneity in structures of casein is complicated by their genetic polymorphism resulting in several variants for each casein. These variants are characterised by point substitutions of amino acids or by deletions of peptide fragments of varying size or by post-translational modifications such as phosphorylation and glycosylation.

In most patients with cow's milk allergy, a high IgE cross-reactivity occurs between the different caseins of cow's milk and between the whole casein fraction of the milk of ruminant species including cow's, goat's and sheep's milk. and cows' milk (Bernard et al., 1999; Restani et al., 1999). However, IgE response and clinical reaction may also be quite specific and allergic reactions to goat's and ewe's milk without cow's milk allergy were recently described (Ah-Leung et al., 2006).

Other Food Allergen Families

Cysteine proteases

The C1, or papain-like, family are part of a much larger family of cysteine proteases, which were originally characterised as having a cysteine residue as part of their catalytic site. The C1 family was identified as having conserved Gln, Cys, His and Asn residues at the active site and includes many endopeptidases, aminopeptidases, dipeptidyl peptidases, some enzymes having both both exo- and endo-peptidase activities (Rawlings and Barrett, 1993). Sequence comparisons show a high degree of relatedness of the residues surrounding the catalytic site across the family. Thus, in papain, the catalytic residues are Cys-25 and His-159, other important residues being Gln-19, which helps form the 'oxyanion hole', and Asn-175, which orientates the imidazole ring of His-159.

A number of CI protease allergens have been identified including inhalant allergens such as the dust mite allergen Der p 1, a β-expansin which is a major allergen of timothy grass pollen, Phl p 1 (Grobe et al., 2002) and a number of food allergens. Despite these structural similarities the C proteases have

only low levels of overall sequence homology, the dust mite allergen, Der p 1 having only approximately 30% identical to the plant cysteine proteases. Notable food allergens are actindin, the major kiwi fruit allergen (Fahlbusch et al., 1998; Pastorello et al., 1998) and the soybean 34kD (oil body-associated) protein, known variously as Gly m Bd 30K, Gly m 1, or P34. The has been reported as the major allergen involved in soybean-induced atopic dermatitis (Ogawa et al., 1993) it is actually associated with the storage vacoles of soybean (Kalinski et al., 1992). There is evidence that the protease activity of the dust mite allergen Der p 1 is important in its allergenicity as it can cleave the human IgE receptor, CD23 and may thereby ablate the feedback mechanism which normally regulates IgE synthesis (Hewitt et al., 1995; Schulz et al., 1995).

Lipocalins

The lipocalins are diverse proteins sharing about 20% sequence identity but with conserved three dimensional structures characterised by a central tunnel which can accommodate a diversity of lipophilic ligands (Flower, 1996). They are thought to function as carriers of odorants, steroids, lipids, pheremones and other compounds. The majority of lipocalin allergens are respiratory, having been identified as the major allergens in rodent urine, animal dander and saliva as well as in insect s such as cockroaches. The only lipocalin which acts as a food allergen is the cow's milk allergen, β-lactoglobulin (Virtanen, 2001).

Lysozyme family

The O-glycosyl hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Like many of the other protein families described here their three-dimensional structures are better conserved than their sequences. They are grouped by structural similarity into clans, one of which is the glycoside hydrolase family 22 which comprises lysozyme type C and α-lactalbumins which have both probably evolved from a common ancestral protein. However, they have distinctly different functions with lysozyme acting as a muramidase, hydrolysing bacterial cell wall peptidoglycan whilst lactalbumin is involved in lactose synthesis in milk. In addition, unlike lysozyme, α-lactalbumin binds calcium. Two food allergen belong to this clan, the minor hens' egg allergen, lysozyme (Gal d 4) and the minor cows' milk allergen α-lactalbumin having little sequence homology but superimposable three-dimensional structures (Nitta and Sugai, 1989).

Transferrin family

Transferrins are eukaryotic sulphur-rich iron-binding glycoproteins which function *in vivo* to control the level of free iron. They have arisen by duplication of a domain, with each duplicated domain binding one iron atom. They include blood serotransferrin (siderophilin); milk lactotransferrin (lactoferrin); egg white ovotransferrin (conalbumin); and membrane-associated melanotransferrin. Both lactoferrin and ovotransferrin have been identified as minor allergens in cows' milk and egg respectively.

Serpins

The term serpin is derived from the fact that these proteins are SERine Proteinase INhibitors and are present in all groups of organisms with apart from fungi. They are involved in a variety of physiological processes including blood clotting, inflammation amongst many others. Many of the family members have no inhibitory activity but those that do may act as suicide substrate inhibitors, forming acyl intermediates which bind irreversibly to a protease (van Gent et al., 2003). Food allergens belonging to this family are the hens' egg allergen, ovalbumin and Z4 from barley, a beer allergen.

Arginine kinases

Argingine kinase belongs to a family of structurally and functionally related ATP: guanido phosphotransferases that reversibly catalyze the transfer of phosphate between ATP and various phosphogens. They have highly conserved active sites including cysteine residues which may be important in catalysis. They have been identified as allergens in invertebrates including food allergens

in shrimp (Yu et al., 2003), and non-food allergens in indianmeal moth house dust mite, cockroach, king prawn, lobster, and mussel (Binder et al., 2001).

Kunitz inhibitors

The Kunitz/bovine pancreatic trypsin inhibitor family are active against serine, thiol, aspartic and subtilisin proteases. They are generally small (~50 residue) proteins with three intra-chain disulphide bonds stabilising a tightly folded three-dimensional structure. They belong to a superfamily of structurally-related proteins but share no sequence similarity. Members of this family have also been identified as allergens in in cows' milk, Bos d TI and tentatively in *Anisakis simplex* (Shimakura et al., 2004). From the second plant protein family, not related to the animal protein family, food allergens have been identified in soybean (Moroz and Yang, 1980) and potato (Seppala et al., 2001).

Chitinase 1

Chitinases hydrolyse chitin, a major polymer component of fungal cell walls, the cuticles of arthropods and exoskeletons of crustacean and a may therefore play a role in plant protection against pests and pathogens. Based on their sequence and structural homologies they have been divided into six classes with class I chitinases having an N-terminal chitin-binding domain which is homologous with the latex protein hevein. This may explain why class I chitinases from avocado, chestnut and banana have been identified as cross-reactive allergens in the latex-fruit syndrome.

Thaumatin-like proteins

The family of thaumatin-like proteins (TLPs), also designated PR-5 play an important role in the plant's defence and are thought to be produced in response to pathogen infection or to osmotic stress but are also a group of highly stable plant food allergens. Their eight disulphide bridges contributes to their exceptional stability, allowing them to both resist thermal denaturation and digestion (Smole et al., 2008). A number of TLP allergens have been identified in fruit including apple, kiwi, grape and cherry (Breiteneder, 2004).

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ANNEX 3. BIOINFORMATICS FOR THE ASSESSMENT OF ALLERGENICITY/IGE-CROSS-REACTIVITY OF NEWLY EXPRESSED PROTEINS IN GMOS

3.1. Introduction

Atopic individuals have an intrinsic tendency to develop type I hypersensitivity allergic reactions against one or several allergens. Proteins with potential to provoke allergic reactions can be divided into two subcategories, complete and incomplete allergens, *i.e.* those which can educate the immune system (sensitization) to a full response including the induction of immunoglobulin E (IgE) antibodies and those which only have the ability to trigger release of inflammatory mediators through cross-reactive IgE binding, respectively (Aalberse et al., 2001; Vieths et al., 2002; Weber, 2001). The ability of incomplete allergens to elicit allergic reactions in individuals already sensitized to another allergen through cross-reactive IgE-antibodies is mostly due to properties inherent in their structure, *i.e.* they have the ability to mimic conformational or linear IgE-binding *epitopes* on the complete allergens. The ability to initiate the synthesis and induce the secretion of IgE antibodies can, however, not be explained by protein structure alone, since the duration, amount and conditions of exposure (matrix surrounding the allergen, nature of the host tissue, etc) to the allergen probably are just as important factors.

Over the last decade, bioinformatics methods have been widely used for collecting, storing, and analyzing molecular and/or clinical information of importance for allergy (Mari, 2005). Several databases holding information on allergenic proteins, including their amino acid sequence or structural features, are publicly available on the Internet. Some of these web resources also contain platforms enabling the user to subject a query amino acid sequence to computational assessment trials to estimate its level of potential allergenicity, based on algorithms especially designed for this purpose.

A typical *in silico* risk assessment of potential allergenicity minimally requires the following two resources: a repository of all known allergens with determined amino acid sequence and/or 3D structure as well as an algorithm for searching relevant similarity between a query protein and the allergen database. With the purpose of improvement of bioinformatic approaches relative to the current guidelines for *in silico* assessment, the various databases and algorithms, primarily used by different applicants and researchers within this field, are reviewed and discussed.

3.2. Current *in silico* guidelines

In 1996, the joint International Life Sciences Institute – International Food Biotechnology Council (ILSI/IFBC) presented a decision-tree for a comprehensive safety assessment of GM foods in the context of allergenicity, which encompasses several principally dissimilar testing methods including an amino acid sequence comparison for xenoproteins, obtained from sources with known allergenic potential, to allergen sequences (Metcalfe et al., 1996). Several years later, the joint Food and Agriculture Organisation and World Health Organisation (FAO/WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology presented a revised scheme, in which a similar bioinformatics analysis is a mandatory initial step regardless of transgene origin. The recommended *in silico* protocol holds a two-part procedure wherein a warning flag is raised by either a match of six consecutive amino acids or an identity of more than 35 % (as measured with *sequence alignment,* see next section) over an 80-amino acid window of the query protein, in both cases to a documented protein allergen (FAO/WHO, 2001). The first of these FAO/WHO criteria is conducted to identify potential linear IgE epitopes or possibly also T-cell epitopes, whereas the second criterion aims at also detecting potential conformational IgE-epitopes.

The identical peptide match method using a peptide length of six amino acids has attracted much criticism in recent years, since it generates too many false positives in testing of potential allergenicity (Bjorklund et al., 2005; Gendel, 2002; Hileman et al., 2002; Kleter and Peijnenburg, 2002; Silvanovich et al., 2006; Soeria-Atmadja et al., 2006; Stadler and Stadler, 2003). Moreover, even in linear B cell epitopes some amino acids can be replaced without loss of *antibody* binding. Consequently, the Codex Alimentarius Commission did not adopt the criterion of six identical amino acids in their guideline (Codex Alimentarius, 2003), but rather concluded that the scanning peptide size should be based on a scientifically justified rationale. In 2006, the European Food Safety Authority (EFSA) released a guidance which is in line with the recommendations of the Codex Alimentarius as regards the assessment of the allergenicity of GM foods and in which improved *in silico* testing for prediction of potential allergenicity is recommended (EFSA, 2006).

3.3. Allergen online databases

Several reviews on allergen databases have been published during the last five years (Brusic et al., 2003; Gendel, 2009; Gendel and Jenkins, 2006; Mari, 2005; Schein et al., 2007). An excerpt of these publicly available and searchable repositories is listed in Table I. There are large differences between the databases as regards the number of molecules listed as allergens (or isoallergens), as well as the information available on these molecules and their source organism. Most of these publicly accessible online repositories contain information on allergens as well as links or accession numbers to general databases, such as UniProt (Bairoch et al., 2005), in which the actual amino acid sequence of the corresponding allergen can be found and retrieved. Some databases also contain links and accession numbers to structural or domain information on the allergen molecules, whereas other include experimentally verified IgE-epitopes and/or computationally derived *motifs* (a motif can be described as a substructure in a protein that can be connected to function).

The following features of an allergen database are desirable for risk assessment usage:

1. *Selection criteria for inclusion of allergens in the databases should be given.* There are several important issues regarding the database design and quality for usage in development, performance estimation and utilization of *in silico* assessment methods. For example, all listed allergens in the databases may not be clinically relevant (some may have been included solely because they have an IgE-binding post-translational modification, such as *cross-reactive carbohydrate determinants*) and certain allergens may only occur as protein fragments lacking the amino acid positions crucial for their allergenicity. Currently, the selection criteria of entering allergens into most on-line databases are not fully transparent. Thus, reasons for molecules appearing in the database could range from information on structural similarity to known allergens to documentation on binding to IgE-antibodies in individuals allergic to their source. Proteins, with established allergenicity/IgE-cross reactivity but founded on weak documentation, should be excluded during development and validation of new *in silico* methods. In risk assessment trials, on the other hand, the aim is to identify allergens, thereby reducing the risk of introducing new allergens on the consumer market. Therefore, in actual assessments proteins having weaker documentation on allergenicity/IgE-cross reactivity could possibly be included in the allergen databases even if the risk of contamination of the database with non-allergens increases.
2. *Possibility to directly perform bioinformatics risk assessment using the listed allergens or extract/export data necessary for that purpose.* Reported methods for *in silico* assessment of allergenicity/IgE-cross reactivity are nearly always based on amino acid sequence information. Thus, meaningful usage of an allergen database in risk assessment exercises requires either that such methods are already implemented as tools in the repository or that amino acid sequences easily can be downloaded in appropriate formats (so that they can be used in stand-alone implementations of the aforementioned methods). Several allergen databases contains on-line tools for directly assessing allergenicity/IgE-cross-reactivity of a query protein using various algorithms and criteria based on submission of the corresponding

amino acid sequence (see Annex 3.9). An expedient downloading procedure of (selected excerpts of) these databases is also a preferred feature.

3. *Maintenance and upgrading of databases*. Some of the databases listed in Table I have not been updated for a long time. Continuous curation is, however, important, thereby giving access to recently discovered allergens (being either sensitizing or cross-reactive). Moreover, it is desirable that old versions of databases are stored in an accessible form, thereby facilitating possibility to go back and analyze earlier assessments. Therefore, bioinformatics risk assessments should also be accompanied with date of consultation and/or version number of database.

An allergen database designed specifically for risk assessment should include amino acid sequences of all characterized allergens. To increase the usefulness of the database for future bioinformatics protocols, it should preferably be associated with available molecular information, such as knowledge on epitopes and 3D-structure.. Moreover, information available on quality of documentation on allergenicity/IgE-cross reactivity of the allergen, such as binding to IgE antibodies in allergic individuals, is also valuable. Ideally, all records should be searchable according to what kind of allergenicity documentation there is entailed to it so that users could export datasets tuned to their own quality criteria. The Allergome platform is one of the most comprehensive on-line database available, both as regards the number of characterized molecules, as well as the amount and diversity of information (including documentation on allergenicity) on individual allergens (Mari et al., 2006). Currently, however, Allergome does not allow *in silico* risk assessment of query amino acid sequences. Therefore, risk assessment using this repository requires that all amino acid sequences firs are downloaded and thereafter used as input to a stand-alone assessment tool. Like several other databases (see Table IV), the AllergenOnline database allow comparisons of query proteins according to the 35 % sequence identity criterion (see Annex 3.2). Moreover, inclusion of allergens into the database is supervised by a peer review panel and overall inclusion criteria are stated on the website. Since none of the databases listed in table I is complete, as regards numbers and diversity of allergens, a search against a single database might overlook important similarities to a known allergen. To reduce this risk, bioinformatics risk assessment trials should include searches to several databases.

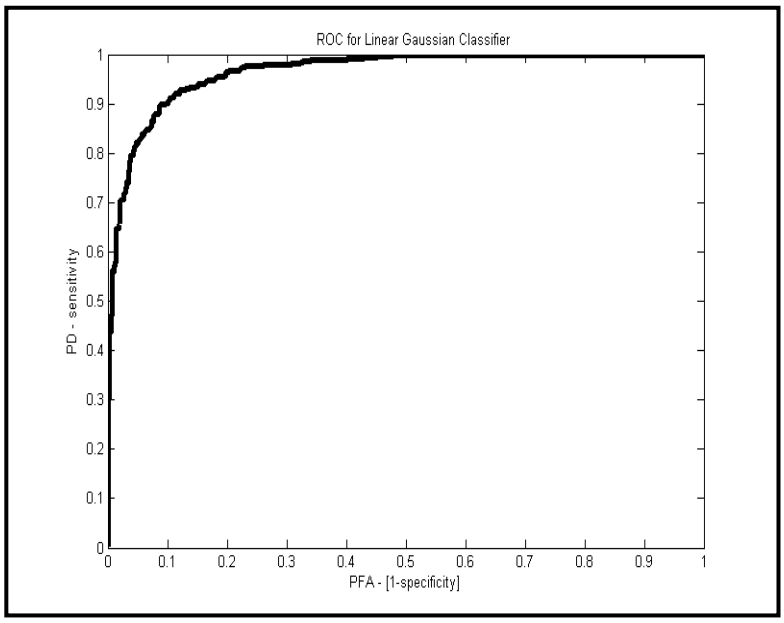
3.4. Performance estimation of *in silico* methods

Accurate prediction of protein allergens using bioinformatics methods would be an invaluable help in the risk assessment of GM foods. A prediction model typically consists of a prediction procedure in combination with a dataset (allergens and sometimes also presumed non-allergens). There are mainly two measures that are used to describe the performance of an allergenicity prediction model; *sensitivity* and *specificity*, which are defined as follows:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

Another performance measure commonly used in several reports instead of specificity is *false alarms* (1-specificity), whereas the term sensitivity is also referred to as *recall*. It is easy to understand that there is a trade-off between sensitivity and specificity. Forcing an algorithm (by changing its detection statistic threshold) to perform with 100% sensitivity *i.e.* all allergens are correctly predicted, will automatically imply an increased risk of generating more false positive results. This trade-off can be visualized using *receiver operating characteristic* (ROC) curves, which depict sensitivity on the ordinate versus the fraction of false positive scores (1 – specificity) on the abscissa, as they vary with incremental alterations of the detection statistic thresholds for a given algorithm.



A simulated example of the shape of a ROC curve

Among the different existing techniques to assess sensitivity and specificity, the two most commonly used approaches in allergen prediction are holdout validation and *k*-fold cross validation (CV). In holdout validation, a set of examples (in this case amino acid sequences) is kept outside all parts of the prediction method design and is only used for testing the accordingly designed prediction system. In *k*-fold CV, all data examples are partitioned in *k* equally sized fractions. In each of *k* iterations, each fraction is used for performance estimation, whereas the other *k*-1 fractions are allowed to design the prediction method. After all k iterations have been conducted, the average sensitivity and specificity is computed. Thus, both techniques evaluates a prediction model, as regards sensitivity and specificity, using test data not earlier used in design of the prediction model.

3.5. Sequence homology bias and its impact on performance

As mentioned earlier, dedicated and publicly available repositories of protein allergens have proven indispensable for the development of computational methods for identifying potentially cross-reactive molecules. An appreciable part of the allergens occurring in these specialized databases are referred to as *isoallergens*. Moreover, a high degree of similarity between allergens may also occur across species boundaries. If an amino acid sequence, occurring in the reference database, has an isoform in the set of test examples used for performance evaluation, the accordingly designed prediction algorithm will easily identify also this isoform as potentially allergenic. Therefore, if redundant sequence datasets are used in the design and performance estimation of a prediction system, there is a large risk of obtaining overly optimistic performance estimates. Although, this issue is well known in most bioinformatics areas it has, however, not yet been extensively discussed in the literature in the context of allergen prediction. Moreover, as reported by Aalberse (2005), it is not clear how many of the hitherto reported algorithms for allergenicity prediction have taken this source of bias into consideration. Non-redundancy is commonly obtained by firstly clustering amino acid sequences so that no examples between clusters share more similarity than a cut-off limit. Thereafter, one representative sequence from each cluster is selected to constitute the non-redundant set. There is currently no general sequence identity redundancy threshold for performance estimation of allergenicity prediction systems but its influence on sensitivity performance was recently described for various computational methods (Soeria-Atmadja et al., 2006). It should be mentioned that redundant databases only impose a problem

during estimation of a method's performance (sensitivity/specificity). Therefore, in the actual risk assessments, redundancy is (for most methods) not an issue.

3.6. Computational methods for risk assessment of potential allergenicity/IgE-cross-reactivity

Most of the methods reviewed below have been developed with the purpose to predict the allergenic potential of a protein. As mentioned earlier, however, *de novo* sensitization is not only determined by the structural properties of the allergen, but other factors are also important. Therefore, computational protocols and algorithms mentioned in the following subchapters could be considered as methods primarily for assessment of IgE-cross-reactivity rather than allergenicity in general.

3.6.1. Methods based on alignment to entire allergen amino acid sequences

Sequence alignment is a method to compare and represent similarities and differences between sequences of biomolecules. It is a fundamental technique in biology since high sequence similarity usually means structural and/or functional similarity. Sequences can principally be aligned in two different ways, globally or locally. Since local alignment aims at finding shorter sequence regions of highly conserved residues, it is preferred for bioinformatics assessment of allergenicity/IgE-cross reactivity. The two most commonly used local alignments methods for searching sequence databases are FASTA (Pearson and Lipman, 1988) and BLAST (Basic local alignment search algorithm) (Altschul et al., 1990), which both are fast approximations of the Smith-Waterman algorithm for optimal local alignment (Smith and Waterman, 1981). A local alignment involves two symbolic sequence representations of DNA or protein arranged next to each other so that their most similar elements are juxtaposed. Every element in the trace of an alignment is a gap, match or mismatch. Matches and mismatches involve alignment of two identical and different amino acid residues, respectively, whereas a gap represents a deletion/insertion in one of the amino acid sequences.

```
Sequence 1: -IRASAGFDL--AGVHYYVTA
             || | ||||  |||| |||
Sequence 2: HIRSS-GFDLLVAGVHTYVT-
```

The example above contains five gaps, marked with '-', several matches, marked with '|', and a few mismatches (empty space between the two sequences). Two major (often user-defined) parameters affecting an alignment procedure of two amino acid sequences are gap penalty setting and substitution matrix, both being important in guiding the algorithm to indicate matches, mismatches or gaps. The former parameter includes both a penalty for opening a gap, as well as for extending it, and is implemented to avoid excessive insertion of gaps in the alignment. Substitution matrices contains scoring values for aligning two amino acids to each other, wherein matches (of two identical amino acids) typically correspond to high scores, whereas mismatches are assigned low values. The category of substitution matrices based on evolutionary analysis of related proteins families, such as the BLOSUM series, have different grading on mismatches, where those involving two evolutionary similar amino acids are assigned higher scores than others. The impact of alignment parameter setting (gap penalties and substitution matrix) on the FAO/WHO alignment recommendation has not been extensively studied. As regards gap opening penalty, it has been concluded that the change of FASTA default value from 12 to 10 did not significantly alter the results (Ladics et al., 2007). Although not evaluated, it is likely that similar conclusion could be drawn as regards choice of substitution matrix, at least if default substitution matrices of different local alignment tools are considered (such as BLOSUM50 or BLOSUM62). Thus, until a specific alignment parameter setting has been proven to outperform other alternativs (based on proper evaluations), use of default alignment parameter settings should be adequate for risk assessment.

As mentioned earlier, current bioinformatics protocol for IgE-cross-reactivity testing recommends identity of more than 35 % over an 80-amino acid window of the query protein to a known protein

allergen as a criterion for further testing. The proposed procedure for analysing similarity involves an initial segmentation of the query protein into a complete (overlapping) set of 80 amino acid long sequences. Thereafter each 80-mer is compared to an allergen database using the local alignment tool FASTA to reveal the best alignments (FAO/WHO, 2001). Finally, these outputs are analysed to identify matches that meet the abovementioned similarity criterion. There are several different methods to calculate the crude measure percent identity (PID) from an alignment of two amino acid sequences (Raghava and Barton, 2006), where some methods neglect gaps and others treat them as mismatches. If gaps are neglected in the calculation, a heavily gapped alignment (whih often indicates poor similarity) can still return an overly high PID. If such an alignment would be evaluated by a PID calculation method considering gaps as mismatches the PID will decrease, which better reflects the poor similarity between the two sequences. The sequence percent identity would thus be calculated as follows:

$PID = \frac{Identical}{Aligned + Gaps}$, where *Identical* is the number of identical positions in the alignment, *Aligned* is the number of all aligned positions in the alignment (including mismatches) and *Gaps* is the number of inserted gaps in the alignment.

The identity limit of 35 % is considered conservative, since allergenic cross-reactivity usually requires more than 50-70 % sequence identity (Aalberse, 2000) although exceptions to this rule-of-thumb exist. Moreover, a recent report by Ladics et al., (2007) disputes the 80-mer sliding window approach, since it can generate both more false positives, as well as less statistically significant alignments than results derived from a database search with the entire query amino acid sequence only. The former issue was demonstrated by comparing the assessment results of the two procedures, when tested with different datasets of presumed non-allergens. Since FASTA searches, using the entire query amino acid sequence, overlook some of the presumed false positives identified with the sliding-window 80-mer search, it is reasonable to argue that the former approach is more specific. The second criticism against the sliding window approach relates to the statistical significance of the resulting top alignments. An alignment derived from a FASTA search of a database is accompanied with an E()-value, which represent the number of times the corresponding alignment score is expected at chance. FASTA alignments with the entire query amino acid sequence corresponded to lower E()-values than those using the 80-mers, thereby having higher statistical significance. On the other hand, the identified alignments obviously meet the criterion of at least 35 % identity over a window of minimum 80 amino acids. From that perspective, the conservatively set sequence identity criterion rather than the 80-mer approach is responsible for the false positives. Moreover, it is plausible that some of these false positives also would have been recognized, using alignment of the entire query sequence, at a slightly lower sequence identity threshold. Nonetheless, the added value of initial amino acid sequence segmentation into overlapping 80-mers prior to database searches is questionable. This approach may be more appropriate if the query protein has (or is predicted to have) a multi-domain structure, since a single domain with similarity to a known allergen theoretically could escape detection if inserted into an otherwise non-allergenic protein.

Regardless sequence identity threshold and method for searching similarity (either using the entire amino acid sequence or the complete set of the corresponding 80-mers) it should be noted that matches slightly below the limit may be just as important as those slightly above. Thus, the sequence identity threshold should be used as guidance rather than strictly discriminatory for further testing. Moreover, it would be valuable to evaluate a match between a query protein and allergen more quantitatively than the simple categories “above limit” or “below limit”.

Another core issue, not yet raised in the literature, is the use of PID as a discriminatory criterion in IgE-cross reactivity risk assessment. According to the current recommendations this criterion equals to occurrence of 35 % pairs (or more) of identical amino acids over a window of at least 80 amino acids in the top alignments to allergens obtained with FASTA. The initial FASTA searches, based on evolutionary substitution matrices (being the default setting of most local alignment tools) are, however, set to identify the best alignments according to a different criterion, since also partial

matches (evolutionary similar mismatches) are assigned high scores (see above). Therefore, the resulting alignments are optimized to include pairs of both identical and similar amino acid residues. In the following procedure, however, only the identical matches are included, whereas the high-scoring partial matches (being mismatches of similar amino acids) of the alignment are ignored. Thus, since evolutionary substitution matrices are used to find best alignments of a query protein to an allergen database, the criterion in the subsequent analysis of the alignment should be analogously based, *i.e.* on sequence similarity rather than sequence identity. Such criteria could, for example, be based on other local alignment output, such as Z-scores or E()-values. In a recent report, use of a threshold based on method to calculate an E-value for assessing query alignments to allergens is suggested, which results in fewer false positives among corn proteins when compared to application of the 35% identity criterion (Silvanovich et al., 2009). Further research and evaluations of more and larger datasets may prove this threshold (or alternative thresholds also based on sequence similarity) being superior to those founded on percent identity.

Apart from the recommendations by FAO/WHO and Ladics et al. (2007) several other procedures have been suggested (Gendel, 1998b; Soeria-Atmadja et al., 2004; Zorzet et al., 2002), which are also founded on features from sequence alignment procedures against *entire* amino acid sequences of allergen proteins. In the first of these papers, Gendel suggested an initial alignment search using an identity matrix, followed by an additional search using either a biochemical or an evolutionary substitution matrix. The two latter papers describe the use of supervised learning algorithms, which are trained to discriminate between alignment-based features typical for allergens and presumed non-allergens, respectively.

Although many allergens appear to cluster into relatively few protein families (Aalberse et al., 2001; Breiteneder and Ebner, 2001; Jenkins et al., 2005; Radauer and Breiteneder, 2006), most members of such protein families seem to be devoid of allergenic properties (Mills et al., 2004). Therefore, there is a risk that algorithms searching for similarities against an allergen database of entire amino acid sequences may find similarities characteristic for other functional features than IgE-cross reactivity.

3.6.2. Methods based on similarity to computationally generated motifs/peptides from amino acid allergen sequences

To reduce false positives due to similarity matches against parts of the allergens unimportant for allergy, several recent studies have focused on the construction of algorithms for automated motif generation. The purpose of these algorithms is to create a peptide set, wherein only motifs common for allergens are supposed to be included. Thereafter, a similarity search is performed between the query protein and the allergen motif set, which replaces the original dataset of entire allergen amino acid sequences. Stadler and Stadler have reported the iterated use of the Multiple EM for Motif Elicitation (MEME) algorithm (Bailey and Elkan, 1994) to generate motifs that are present within two or more allergens (Stadler and Stadler, 2003), whereas Li *et al.* (2004) have presented an alternative algorithm, in which the motif-finding method is founded on wavelet analysis. Since both these approaches fail to extract motifs for 10-20 % of the allergens, an add-on procedure has also been suggested, wherein a similarity search (using alignment) against the entire amino acid sequences of these (unmatched) allergens is performed. In a recent report, Kong *et al.* (2007) described a procedure that uses a combination of several MEME-derived motifs, which showed higher specificity than employing single motifs only. Mari *et al.* (2006) showed that generated motifs may, in some cases, be useful for identifying IgE epitopes.

Although algorithms developed to solely recognize common inter-allergen motifs might return an overall reasonably good prediction performance in regular test procedures, there is still a risk that they target motifs specific for protein family although these motifs have little or no relevance to allergenicity/IgE-cross-reactivity. This issue is most important in those cases where protein allergens have relatively closely related human homologues. For example, in the study by Li *et al.* (2004) it is stated that some of the motifs generated from allergen tropomyosins are specific to the tropomyosin

family itself rather than the allergen counterparts. Hence, algorithms solely searching for motifs that are common in allergens may suffer (although to a lesser content) from the same problems as those using similarity searches in entire amino acid sequences. As an alternative to algorithms searching for inter-allergen motifs, Björklund *et al.* (2005) have reported an algorithm based on a novel principle, wherein allergen-representative peptides are obtained by selecting peptide sequences of allergens that occur infrequently in presumed non-allergens. A drawback with this method is that it is restricted to choose the same amount of peptides from each allergen and that the obtained peptides are constrained to having the same length. These two issues were addressed in the refinements of this method(Soeria-Atmadja et al., 2006). The latter method was also able to discriminate between allergens and presumable non-allergens in tropomyosin and parvalbumins protein families. A drawback with both of the latter methods is that they require a dataset of presumable non-allergens to select the peptides (see Usage of negative examples in modeling and evaluation of *in silico* methods).

3.6.3. Methods based on similarity to experimentally verified IgE-epitopes

As mentioned earlier, the identical peptide match criterion, using 6 amino acids long peptides as proposed by FAO/WHO, has been substantially criticized for being too unspecific. In order to increase the specificity, Kleter and Peijnenburg (2002) have proposed a 2-step strategy wherein the positive outcomes, as revealed by the aforementioned criterion, are further screened for the presence of potential linear IgE-epitopes. This approach includes comparison of these peptides with known IgE-epitopes and/or evaluating their potential antigenicity with computational methodology (see epitope prediction section).

Saha and Raghava (2006) evaluated the use of similarity to experimentally verified IgE-epitopes for prediction of allergenicity/cross-reactivity and found that 11 % of an independent allergen data set could be correctly assigned. Thus, at the present time there are too few characterized IgE-epitopes for these methods to be sensitive enough as stand-alone *in silico* testing procedures, but may be useful as complement to other bioinformatics algorithms.

3.6.4. Methods based on amino acid composition or physico-chemical properties

An interesting alternative to both the identical peptide match method, as well as alignment criteria, has been proposed by Ivanciuc *et al.* (2002). Amino acid sequences are firstly transformed into a numerical representation based on five-dimensional physico-chemical descriptors of amino acid properties (Venkatarajan and Braun, 2001), and similarity between the numerical vectors is thereafter calculated as Euclidean distance. Although this method may be useful for the search of potential epitopes in known allergens (Schein et al., 2005), it has not yet been sufficiently evaluated in the context of risk assessment of a query protein's allergenic potential. In a report, Saha and Raghava (2006) suggested (among several other methods) the use of amino acid composition and dipeptide composition as features in combination with supervised machine learning for prediction of allergenicity/IgE-cross-reactivity. This approach did, however, show low specificity when evaluated with Swiss-Prot.

Recently, Cui *et al.* (2007) presented a method based on supervised machine learning in combination with sequence-derived structural and physicochemical properties by using different *propensity scales* (see B-cell epitope prediction). More specifically, proteins are converted into numerical vectors based on their amino acids' hydrophobicity, normalized Van der Waals volume, polarity, polarizability, charge, surface tension, secondary structure and solvent accessibility. The authors describe global (over the whole protein) composition of each of these properties using three descriptors, firstly introduced by Dubchak *et al.,* (1995) for predicting protein-folding class. This approach seems very promising since it yields overall good accuracy but foremost since it correctly assigns also several allergens lacking sequence similarity to other allergens. A possible drawback, though, is that currently no information is given on which of the known allergens the query is most alike. Thus, this makes it difficult for a risk assessor to further investigate the allergenic potential through *in vitro* or *in vivo* methodology.

3.6.5. Usage of negative examples in modeling and evaluation of in silico methods

As mentioned earlier, dose and route of exposure, which may be just as important as characteristics inherent to the protein, are not considered in bioinformatics testing. Thus, a change of the quantity of a protein in a GM-food could be sufficient to change its allergenic potential. Therefore, screening for true non-allergenicity of a protein is currently not possible. Nevertheless, a dataset of presumed negative examples (non-allergens) is a prerequisite for estimating specificity (see above). Moreover, some of the prediction methods mentioned above, also requires the use of presumed non-allergens in either the procedure for generating motifs/peptides or training a supervised learning algorithm. Accordingly, in the following section the term "presumed non-allergens" refers to proteins with presumably low allergenic potential under normal conditions/exposure.

In the reports by Stadler and Stadler (2003) and Li *et al.* (2004), presumed non-allergens, used as one way of measuring specificity, are simulated by shuffling the order of amino acid positions in allergen protein sequences. It is, however, unclear if these virtual amino acid sequences are representative for non-allergens or even for proteins. In the study by Björklund *et al.* (2005) presumed non-allergens were selected from several commonly consumed commodities, a dataset that is also used by Saha and Raghava (2006). Since most of these proteins were of plant origin the dataset is probably not representative for *all* presumed non-allergens. Moreover, for some of the proteins, such as those belonging to the rice proteome, it is very uncertain if they are good candidates as negative examples. The risk of allergens contaminating the non-allergen dataset should be considerably higher in the work by Furmonaviciene *et al* (see "Future studies"), since the presumed non-allergens are homologous to known allergens. In the report by Soeria-Atmadja *et al.* (2006), the human proteome (with some exceptions) is employed as a negative filter to generate peptides presumably important for allergenicity/IgE-cross-reactivity. Cui *et al.*, (2007) suggested very recently an interesting procedure to obtain presumably non-allergens to be used in their evaluation. Protein families in the Pfam database (Finn et al., 2006) that are absent of any documented allergens are selected and representative members from human, bovine, chicken, pear, apple, peanut (and some others) are then chosen as presumed non-allergens. Another important aspect is that none of the abovementioned methods for selecting presumable non-allergens have considered their abundance in their source, which is an important factor as regards allergenicity.

3.6.6. Comparison of performance estimates for different methods

Since the amount of allergen that can be sued as examples is relatively scarce, k-fold CV has been the most commonly used method for sensitivity evaluation of an allergenicity/IgE-cross-reactivity prediction method (Bjorklund et al., 2005; Li et al., 2004; Stadler and Stadler, 2003; Zorzet et al., 2002), although several of the most recent studies have used an independent holdout test set (Cui et al., 2007; Saha and Raghava, 2006; Soeria-Atmadja et al., 2006). CV has also been used to reveal the predictor's estimated specificity (Li et al., 2004; Stadler and Stadler, 2003; Zorzet et al., 2002). The most thorough procedure for specificity assessment, however, is to estimate the ratio of allergens in the entire SwissProt database (Cui et al., 2007; Kong et al., 2007; Li et al., 2004; Saha and Raghava, 2006; Soeria-Atmadja et al., 2006; Stadler and Stadler, 2003).
Table II lists studies where both sensitivity and SwissProt estimation have been assessed. Some of the aforementioned methods have been compared with the two FAO/WHO criteria mentioned earlier. In only a few studies new algorithms have, however, been bench-marked to each other. Since the performance of the algorithms reviewed here has been estimated using different datasets, it is difficult to discriminate between them.

3.6.7. B-cell epitope prediction algorithms

Various methods exist to predict the parts of the protein molecule that are likely to be antigenic and recognized by antibodies, *i.e.* epitopes. These methods do commonly not discriminate between the

different classes of immunoglobulin antibodies, such as IgE, IgA, IgG, or IgM. Epitope prediction methods can be divided into two subcategories: those that use the linear amino acid sequence of the protein as input and those that consider the three-dimensional structure of the protein (Greenbaum et al., 2007).

The classical way of predicting linear B-cell epitopes is by the use of *propensity scale* methods, which assign a propensity score to every amino acid, based on studies of their physico-chemical properties. These methods usually use a sliding window of a fixed number of contiguous amino acids, for which, at each step of sliding, separate propensity scores are combined or averaged into a score assigned to a specific point within the sliding window (Hopp and Woods, 1981). Most propensity scales are based on the hypothesis that amino acid residues on the surface of the three-dimensional structure of a protein molecule are more accessible to antibodies and therefore more prone to binding. Hydrophilicity of the amino acids indicates the likelihood that will they are exposed at the protein surface to the aqueous environment, whilst hydropathicity indicates the likelihood that residues will be buried inside the hydrophobic core of a protein. Another, more empirical approach is to consider the relative frequency with which specific amino acids have been observed to occur at the surface of known three-dimensional protein structures. It should be borne in mind, though, that some epitopes of allergens are known to occur in the inside of proteins, where they become accessible after denaturation or protein degradation into peptides. Moreover, Blythe and Flower have evaluated the performance of 484 propensity scale methods for B cell epitope prediction and found that even the best performing methods could marginally outperform random prediction (Blythe and Flower, 2005).

Another approach is to consider the surface of the three-dimensional structure of a protein, if available, and to identify those residues exposed on the surface that are accessible to antibody binding. Groups of accessible residues that occur within a confined area of the surface that can interact with an antibody binding site are considered epitopes. In contrast to the linear-sequence-based methods, this approach pertains to non-linear, discontinuous epitopes. In addition to the surface of the antigenic protein, also knowledge about residues involved in specific antibody-protein interactions can be exploited, such as derived from crystallographic data on the bound complex or derived using mimotope technology. For more information on 3D-structure-based B-cell epitope prediction, see Greenbaum *et al* (2007).

3.7. T-cell epitope prediction algorithms

There are several bioinformatics tools available for identification of T-cell epitopes to certain specific MHC alleles including those of MHC class II, such as MULTIPRED (Zhang et al., 2005), SYFPEITHI (Rammensee et al., 1999), EpiMer (Meister et al., 1995) and TEPITOPE (Sturniolo et al., 1999). Moreover, T-cell epitope prediction methods are considered more reliable, as regards both specificity and sensitivity, than those designed for identification of B-cell epitopes. Prediction of one or several peptides of the query amino acid sequence as being potential MHC class II binders indicates, however, immunogenicity in general, rather than allergenicity. It is doubtful whether these algorithms can predict T cell epitopes being specific for the proliferation of T_H2 cells, which are associated with allergic sensitization. Therefore, it is currently not straightforward how results from *in silico* T cell epitope predictions should be viewed upon in risk assessment of allergenicity.

3.8. Future studies

Since there are relatively few allergens with determined tertiary structure as compared to those with known primary structure, most efforts in allergenicity prediction has been founded on similarity searches on the amino acid sequence level. There has, however, been much research recently aiming to reveal correlations between structure and protein allergenicity rather than to create a prediction system for allergenic potential (Barre et al., 2005; Furmonaviciene and Shakib, 2001; Jenkins et al., 2005; Johannessen et al., 2005; Neudecker et al., 2001; Roy et al., 2003; Schirmer et al., 2005). A recent report by Furmonaviciene *et al* (2005) describes the employment of the ConSurf server (Glaser et al.,

2003), which is founded on phylogenetic relationships between sequence homologues, to identify functionally important regions of the surface on allergens with known 3D structure. In analogy with the work of Björklund *et al* (2005), presumed non-allergens belonging to the same families as the allergens, have also been included to prevent generating motifs specific for protein family rather than allergenicity. Although this approach is very interesting, no suggestions how it could be used as a prediction tool has been presented. Furthermore, as mentioned earlier, there are so far relatively few allergens where the 3D structure has been determined. As more structural information on allergens is revealed, the search for common structural motifs is, however, likely to improve the quality of assessment of IgE-cross-reactivity and allergenicity. Moreover, different state-of-the-art algorithms for structure prediction (Petrey and Honig, 2005), applied on allergens, which only have their amino acid sequence determined, could further improve prediction accuracy. For example, a two-step protocol to identify potentially cross-reactive peanut-lupine proteins has been described that firstly performs FAO/WHO *in silico* criteria and thereafter a visual comparison of the matching sequences' predicted three-dimensional structure models (Guarneri et al., 2005). The described procedure needs substantial refinements before it could be used as a bioinformatics risk assessment tool, such as implementing it as an automated process, providing a computational measurement for describing relevant structure similarities, and most importantly, it must be validated using the methods described earlier (see Annex 3.4).

3.9. Bioinformatics tools available over the Internet

3.9.1. Risk assessment based on FAO/WHO guidelines

Table III lists websites where a search using the complete or parts of FAO/WHO bioinformatics protocol can be carried out, although each website uses different repositories as the allergen reference dataset. Some of these servers hold only the 35 % sequence identity criterion, revealed using alignment of whole query amino acid or of its corresponding set of overlapping peptides, whereas other also include the identical peptide match criterion.

3.9.2. Risk assessment based on similarity to generated motifs/peptides

Alternatives or complements to FAO/WHO-based bioinformatics risk assessment tools are listed in table IV. The prediction method based on motifs generated by wavelet analysis, as described by Li et al (2004), is publicly available on the WebAllergen server (Riaz et al., 2005), whereas both ADFS (Nakamura et al., 2005) and AlgPred (Saha and Raghava, 2006) hold a prediction method based on the MEME motif discovery tool similar to that reported by Stadler and Stadler (Stadler and Stadler, 2003). The aforementioned AlgPred web server also offers the possibility to predict IgE-cross-reactivity through similarity searches to experimentally verified IgE epitopes, either as a separate method or in conjunction with additional methods. Other prediction algorithms available at this server include the supervised machine-learning methods based on either amino acid or dipeptide composition over the entire protein's amino acid sequence, of which the former can be accessed also at AllerTool (Zhang et al., 2007). EVALLER (version 2.0) is an executable on-line implementation of the principles presented by Soeria-Atmadja *et al* (2006) in conjunction with allergens of the FARRP AllergenOnline database and is available at the Swedish National Food Administration (Barrio et al., 2007; Bongcam-Rudloff et al., 2007). The web server Allergen Protein Prediction E-Lab (APPEL) holds one of the most recent prediction methods, which is founded on supervised machine-learning in combination with a global description of the protein based on amino acid propensity scales (Cui et al., 2007).

3.9.3. Other tools that could be useful for risk assessment

There are also other bioinformatics tools that are not directly focused towards predicting IgE-cross-reactivity/allergenicity potential of proteins but that still may be useful for *in silico* risk assessment. For example, SDAP offers the possibility to do a search with a known epitope to reveal epitope candidates among the allergens available at the server, either using an identical match as criterion or a

match based on similarity, whereas Allergome, AllerTool and ALLERDB hold a visual tool to display graphical representation of allergens known to be cross-reactive.

3.10. *In silico* prediction of the allergenicity potential of open reading frames (ORFs)

Risk assessments of genetically modified plants must also consider the possible formation of short peptides, being the result of translated small open reading frames (ORFs). These putative peptides are often very short (typically shorter than 40 amino acids), and are therefore not readily analyzed with the FAO/WHO alignment criterion. Even though the FAO/WHO identical peptide matching has attracted a lot of criticism for risk assessing full length protein sequences, this method may be applicable to assess ORFs. Thus, if an ORF shares an identical peptide of 8 (or even 7) amino acid residues with any allergen, this ORF should be subject to further evaluation. Presence of contiguous identical peptides as criterion is sensitive to gaps, i.e. an insertion or deletion of an amino acid residue in any of the amino acid sequences. Since insertion/deletion of one amino acid within an epitope does not automatically mean inhibition or reduction of IgE-binding, it is important that the method of choice also can handle gaps. A scientifically more sound in silico analysis would be to perform similarity searches (using alignment) to databases of experimentally verified IgE epitopes or to motifs common in allergens. However, evaluations of resulting high-ranked alignments can not be conducted using the criterion for full length protein sequences, i.e. 35% identity over 80 amino acids. Since the ORFs can differ much in length relative to each other, it is difficult to set a fixed criterion for the evaluation of alignments (between ORFs and known allergens). Therefore, judgement on high similarity of an ORF to an allergen must at the present time be performed case by case. An alternative method to the identical peptide match approach is the peptide similarity tool available at the SDAP server, which is based on similarities of physico-chemical properties rather than simple identity. It is, however, not clear if this peptide matching method can also compensate for gaps.

3.11. *In silico* prediction of potential involvement in coeliac disease

Coeliac disease is an autoimmune disorder of the small bowel that occurs in genetically predisposed individuals. The immunological reaction is caused by prolamins, which are storage proteins rich in proline and glutamine, such as gliadin and hordein from wheat and barley, respectively. The proline-rich peptides that are released by the enzymatic action of proteases during digestion are recalcitrant to further breakdown and are thereby able to reach the immunesystem of the gut mucosa. Upon exposure to these proteins, the body's immune system cross-reacts with the enzyme tissue transglutaminase, causing an inflammatory reaction. In addition, the enzymatic action of transglutaminase itself also deamidates specific glutamine residues, yielding glutamic acid residues, that further increase the sensitization capacity of the coelic-disease-associated proteins. Currently, the only effective treatment is a gluten-free diet.

Although the BIFS database has listings of gluten-associated proteins there are so far no *in silico* prediction procedures publicly available over the Internet. Literature indicate that a limited number of common protein motifs of gluten proteins, including the α-, γ-, and ω-gliadins and low-molecular weight glutenins, are involved with the sensitization of T-cells. These motifs have so far been used for screening amino acid sequences of gluten proteins in cereals for their presence and linkage with potential celiac-disease-causing properties (van Herpen et al., 2006).

3.12. Conclusions and recommendations

Computational screening for potential allergenicity/IgE-cross reactivity is both expedient and inexpensive, compared to laboratory experimentally studies, since the only requirements are a computer and, as mentioned earlier, a feasible algorithm and data repository. Moreover, many of these algorithms accomplish good discrimination between known allergens and proteins presumed to have a lower allergen potential. It should, however, be stressed that all the various computational algorithms available (and reviewed here) are designed to search for (presumed) allergenicity features that are

inherent in the protein's sequence/structure, whereas external factors, such as exposure or post-translational modifications are not taken into account. These algorithms are therefore generally well suited for predicting cross-reactivity but currently not for identification of *de novo* sensitization potential, which is a much more challenging task. Accurate prediction of the latter feature requires more knowledge on the primary sensitization procedure and possibly also further algorithmic refinement to include exposure data in the models.

If computational testing suggests the protein of interest being a potential allergen, further testing, which may be of *in vitro* or *in vivo* character, should be performed in the risk assessment procedure.

3.12.1. Employment of allergen databases

Conclusions

Several reported allergen databases, as outlined in specific and review-type articles, are accessible through the Internet. When used for risk assessment purposes such repositories should be as comprehensive and quality assured as possible. Generally, criteria for inclusion of allergens into databases, are, however, rarely stated and therefore the quality of most databases is difficult to assess. Moreover, none of the existing databases are complete since they all contain errors, as regards presence of presumed non-allergens, as well as absence of true allergens. For example, it is plausible that a significant number of "minor" allergens have not yet been identified and characterized. On the other hand, IgE binding to some among the listed allergens is mainly due to post-translational attachment of glycans, rather than the protein itself. Other important features of databases include good technical maintenance, regular curation, as well as a user-friendly retrieval system so that amino acid sequences can be easily extracted from the databases.

Recommendations

To minimize the risk of overlooking potential IgE-cross reactivity due to incomplete databases, *in silico* consultation of several such repositories should be performed.

Allergen databases used for the assessment of the risk of cross reactivity should be as comprehensive and quality assured as possible. A list of databases appropriate for risk assessment should be established and regularly updated by an official, independent, expert scientific committee. The quality, completeness and reliability of the databases are major issues.

In order to both assure quality of the allergen databases as well as simplifying their use in risk assessment trials, the following features are desirable:

- clearly stated and scientifically sound criteria for inclusion/exclusion of allergen amino acid sequences into allergen databases. These criteria should preferably be established by experts on allergology, molecular biology, biochemistry and bioinformatics.
- continuous curation with respect to allergen information. Moreover, older versions of databases should be stored to enable control of old bioinformatics risk assessments
- simple and user-friendly extraction of allergen amino acid sequences from databases.

3.12.2. Amino acid sequence based bioinformatics protocols

Conclusions

As mentioned earlier, bioinformatics prediction methods of potential protein allergenicity/IgE-cross reactivity is an initial screening step before *in vitro* or *in vivo* testing. Over the last decade, various bioinformatics methods for the abovementioned purpose have been developed. In the review of state-of-the-art, only methods that have undergone some standard bioinformatics (large-scale) performance estimation regarding specificity and sensitivity have been considered. Peptide match of complete identity over 6 contiguous amino acids to known allergens is associated with very poor specificity (many false positives), which has been reported in numerous studies. Moreover, the relevance of an

identical match criterion, regardless of length, is doubtful, since replacement of one amino acid within a potential epitope does not necessarily imply loss of reactivity. The alignment based criterion using a sliding window of 80 amino acids as proposed by FAO/WHO, is still accepted as reasonably adequate also when compared to novel approaches for allergenicity/IgE-cross reactivity prediction. The 35% identity cut-off level may seem overly conservative and 50% identity cut-off has been suggested. Nonetheless, significant cross-reactivity can occur well below 50% identity. The relevance of the sliding window approach as opposed to alignment of the full-length query protein has been disputed in the literature. The former approach seems to be more conservative, in the sense that more amino acid sequences meet the 35% sequence identity criterion using this method. A special concern is attached to the sequence identity criterion, since this strategy neglects partial matches (evolutionary favourable substitutions). Therefore, alternative criteria to assess similarity may prove more useful. In recent years, a range of enhanced algorithms, founded on more advanced principles have been reported. Several among those are associated with both high sensitivity and favourable specificity and are also publicly available over the Internet. A selected fraction of these tools are therefore well qualified to complement the alignment-based method, as suggested by FAO/WHO. Moreover, a decision has to be made on the acceptability of false-negative rate since an overly strict adherence to sensitivity will result in an unreasonable number of false positives (sensitivity versus specificity), without completely avoiding all cross-reactivity risk.

Recommendations

The alignment-based criterion involving 35 % sequence identity to a known allergen over a window of at least 80 amino acids is considered a minimal requirement for risk assessment, although the identity threshold is conservatively set. This procedure could be conducted as follows:

- Alignment using full-length amino acid sequence of query protein to an allergen database (or rather several databases as mentioned earlier).
- As regards choice of the alignment parameters substitution matrix and gap penalties, default settings can be employed (e.g. BLOSUM 50 as substitution matrix, range of 10-12 as gap opening penalty and 2 as gap extension penalty).
- Calculation of percent identity (PID) should be perfomed with gaps *i.e.* inserted gaps are treated as mismatches. $PID = \frac{Identical}{Aligned + Gaps}$, where *Identical* is the number of identical positions in the alignment, *Aligned* is the number of all aligned positions in the alignment (including mismatches) and *Gaps* is the nuber of inserted gaps in the alignment.
- For alignments where the alignment length (Aligned + Gaps) is shorter than 80 amino acid residues, sequence percent identity should be recalculated to an 80-mer window.
- Since matches slightly below 35 % may be just as important as those slightly above, the threshold value should be used as guidance rather than strictly discriminatory for further testing.

Recommendations for further development

More research is required to reveal if there are more favourable alternatives for assessing sequence similarity than sequence identity.

Complementary methods could also be considered to further ensure absence of similarity to known allergens. For example, several web servers that rely on novel principles (based on motifs and peptides specific to allergens) have shown to be highly specific without loosing in sensitivity.

When novel bioinformatics methods are proposed, they should preferably be compared with other methods by means of adequate performance estimation procedures using the same datasets.

3.12.3. In silico prediction of allergenicity potential of open reading frames (ORFs)

Conclusions

The FAO/WHO alignment criterion (35% identity over 80 amino acids) is not suitable for the risk assessment of the short open reading frames (ORFs) that could be translated to peptides due to their small size. If an ORF shares an identical peptide of 8 (or even 7) amino acid residues with any allergen, this ORF should be subject to further evaluation.

Recommendations

Possible inroads for assessing cross-reactivity or allergenicity potential of these short sequences include: a) Alignment to experimentally verified IgE epitopes or to motifs common in allergens. Judgements on whether the resulting alignments show sufficient similarity between ORF and an allergen must be performed case by case; b) Search for identical contiguous peptides of length 8 (or at shortest 7) to known allergens; or alternatively, a search for contiguous peptides (of length 8 or longer) that are similar to known allergens using the peptide-similarity tool at SDAP server, which is based on similarities of physico-chemical properties rather than simple identity.

3.12.4. Future bioinformatics methodology

Conclusions

Even though epitope prediction algorithms may add information as a part of an *in silico* weight of evidence, no reports on performance studies on specificity and sensitivity in terms of allergens have been presented. At the present time, B-cell epitope prediction is seemingly not yet suitable for risk assessment. Moreover, even though accurate T cell prediction algorithms may identify potentially immunogenic peptides, they are currently not directed to allergic sensitization, specifically. Therefore, T-cell epitope prediction may be more suitable for assessment of immunogenicity, whereas the predictive value for allergic sensitization is limited. However, in combination with cellular based tests, T cell epitope prediction may hold prospects for development to usefulness for immunogenicity assessment. 3D motifs are likely to hold information of higher relevance to IgE-cross-reactivity, compared with linear motifs. Gradually increasing numbers of structurally determined allergens in combination with advanced algorithms for structure prediction should enable searches for such structural motifs. There is not yet any report on thoroughly evaluated protocols for measuring similarity to known allergens on the 3-dimensional level. Algorithms to predict epitopes or structural motifs of importance to allergenicity/IgE-cross-reactivity need further evaluation before these may be recommended for risk assessment.

Recommendations for further development

Future work should focus on the completion of databases with information on 3-dimentional conformation of the allergens and on the development/validation of relevant algorithms to identify structural homology on T cell epitopes.

Since recent studies suggest alternative similarity criteria to the 35% identity for assessing the sequence aligment to an allergen, this need to be further evaluated.

1 **Table I: Allergen online databases**

Database	URL	Type of allergens	Clinical information	Sequence information	Structural information	Epitopes	Domain information	Last update
IUIS Allergen Nomenclature Sub-Committee [1]	http://www.allergen.org/Allergen.aspx	All	No	Yes	Accession names to external website	No	No	On regular basis 2009-05-27
Allergen Online (FARRP)[2]	http://www.allergenonline.com	All	No	Links to external website	No	No	No	Yearly version 9.0 2009-01
IMGT allergen page	http://imgt.cines.fr/textes/IMGTeducation/IMGTlexique/A/AllergensBiochemicalData.html	Food	No	Accession names to external website	Accession names to external website	No	No	2007-02-13
The Allergen Database (CSL)[9]	http://allergen.csl.gov.uk	All	No	Links to external website	No	No	No	Not stated
Allergen Database for Food Safety (ADFS) [3]	http://allergen.nihs.go.jp/ADFS/	All	No	Links to external website	Links to external website	Yes	Links to external website	2009-03-10
Bioinformatics for Food Safety (BIFS) [4]	http://www.iit.edu/~sgendel/fa.htm	All	No	Links to external website	No	No	No	Not stated
The InformAll Database	http://foodallergens.ifr.ac.uk/	Food	Yes	Links to external website	Links to external website	Yes	Links to external website	2006-10-18
Allergome [5]	http://www.allergome.org	All	Yes	Yes + links to external website	Links to external website	No	Links to external website	On regular basis 2009-06-30
ALLERDB [6]	http://research.i2r.a-star.edu.sg/Templar/DB/Allergen/	All	No	Yes + links to external website	Links to external website	No	Links to external website	Not stated
Structural Database of Allergenic Proteins (SDAP) [7]	http://fermi.utmb.edu/SDAP/sdap_src.html	All	No	Links to external website	Yes + links to external website	Yes	Links to external website	2009-06-25
AllerMatch [8]	http://www.allermatch.org/	All	No	Links to external website	Links to external website	No	No	2007-12-21
Allfam database [9]	http://www.meduniwien.ac.at/allergens/allfam/	All	No	Links to external website	Links to external website	No	Yes	2009-05-11
Allergen ATLAS[10]	http://tiger.dbs.nus.edu.sg/ATLAS/	All	No	Links to external website	Links to external website	Yes	Yes	Not stated

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Table II: Studies assessing both sensitivity and SwissProt estimation

Study	Method	Sensitivity (%)	SwissProt estimate (%)
Stadler and Stadler [1]	Similarity to generated motifs (MEME)	86,2	4,0
Stadler and Stadler [1]	Alignment 35% over 80 aa OR peptide match (length = 6)	97,0	67,3
Stadler and Stadler [1]	Alignment 35% over 80 aa OR peptide match (length = 8)	92,2	8,0
Li *et al* [2]	Similarity to generated motifs (wavelet transform)	70,6	3,5
Soeria-Atmadja *et al* [3]	Similarity to selected peptides (FLAPs)	86,6	1,5
Soeria-Atmadja *et al* [3]	Similarity to selected peptides (ARPs)	83,2	3,1
Soeria-Atmadja *et al* [3]	Alignment 35% over 80 aa OR peptide match (length = 6)	96,6	75,4
Soeria-Atmadja *et al* [3]	Alignment 35% over 80 aa OR peptide match (length = 8)	88,9	6,2
Saha and Raghava [4]	Amino acid composition	84,2	43,1*
Saha and Raghava [4]	Dipeptide composition	84,8	38,1*
Saha and Raghava [4]	Similarity to selected peptides (ARPs)	66,6	2,0*
Saha and Raghava [4]	Similarity to generated motifs (MEME)	12,4	3,4*
Saha and Raghava [4]	Similarity to IgE epitopes	10,8	1,8*
Cui *et al* [5]	Physico-chemical properties	93,0	2,9
Kong *et al* [6]	Similarity to combination of motifs (MEME)	75,3	3,8

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Table III: FAO/WHO-based bioinformatics risk assessment tools

Webtool	URL	Peptide match	Alignment
AllerPredict[1]	http://research.i2r.astar.edu.sg/Templar/DB/Allergen/Predict/Predict.html	Yes (length = 6)	BLAST
AllerTool[2]	http://research.i2r.a-star.edu.sg/AllerTool/	Yes (length = 6)	BLAST
Structural Database of Allergenic Proteins (SDAP) [3]	http://fermi.utmb.edu/SDAP/ sdap_who.html	Yes (length = userdefined)	FASTA or FASTA sliding window
AllerMatch[4]	http://www.allermatch.org/	Yes (length = userdefined)	FASTA or FASTA sliding window
Allergen Database for Food Safety (ADFS) [5]	http://allergen.nihs.go.jp/ADFS/	Yes (length = userdefined)	FASTA
The Allergen Database at the Central Science Laboratory (CSL)	http://allergen.csl.gov.uk	No	FASTA
Allergen Online (FARRP) [6]	http://www.allergenonline.com	No	FASTA or FASTA sliding window
Allergome [7]	http://www.allergome.org	No	BLAST
Allergen ATLAS [8]	http://tiger.dbs.nus.edu.sg/ATLAS/	Yes (length = userdefined)	BLAST or BLAST sliding window

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Table IV: Alternatives or complements to FAO/WHO-based bioinformatics risk assessment tools

Webtool	Underlying algorithm	URL
WebAllergen [1]	Similarity to generated motifs (wavelet transform)	http://weballergen.bii.a-star.edu.sg/
AlgPred [2]	Similarity to generated motifs (MEME)	http://www.imtech.res.in/raghava/algpred/
AlgPred [2]	Similarity to selected peptides (ARPs)	http://www.imtech.res.in/raghava/algpred/
AlgPred [2]	Similarity to experimentally verified IgE epitopes	http://www.imtech.res.in/raghava/algpred/
AlgPred [2]	Supervised learning based on either amino acid or dipeptide composition	http://www.imtech.res.in/raghava/algpred/
AllerTool [3]	Supervised learning based on amino acid composition	http://research.i2r.a-star.edu.sg/AllerTool/
EVALLER (2.0) [4, 5]	Supervised learning based on similarity to selected peptides (FLAPs)	http://www.slv.se/templates/SLV_Page.aspx?id=19259&epslanguage=EN-GB
ADFS [6]	Similarity to generated motifs (MEME)	http://allergen.nihs.go.jp/ADFS/
APPEL [7]	Supervised learning based on global physcio-chemical descriptions of proteins	http://jing.cz3.nus.edu.sg/cgi-bin/APPEL

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ANNEX 4. ASSESSMENT OF ALLERGENICITY OF NEWLY EXPRESSED PROTEINS IN GMO'S USING *IN VITRO* AND CELL-BASED TESTS

4.1. Introduction

The guidelines for food safety assessment of GMOs published by Codex alimentarius (2003) recommend various *in vitro* methods as part of the "weight-of-evidence" approach for assessing the potential allergenicity. These methods include the following tests with newly expressed proteins:

- The degradation of the newly expressed proteins by the proteolytic enzyme pepsin in defined conditions (referred to as "pepsin resistance test")
- The binding by newly expressed proteins of IgE-containing sera from patients who are allergic towards an allergen of interest, such as:
    - the source of the transgene, if this happens to be an allergen, or
    - an allergen with which the newly expressed protein shows a relevant degree of structural similarity.

In the following sections, current practice and recently acquired knowledge are considered, and further directions are discussed in this area of allergenicity assessment.

Commonly, given the low expression levels of transgenic proteins in genetically modified crops, recombinant equivalents of these proteins are produced in microorganisms, such as *Escherichia coli*, which are amenable to the production of sufficient quantities. It is this protein which is usually used for *in vitro* tests. Codex alimentarius' and EFSA GMO Panel's guidance requires that these proteins be equivalent to that expressed in the genetically modified crop. This usually entails a comparison between the two different proteins with regard to molecular mass (*e.g.* electrophoretic mobility, mass spectrometry), glycosylation, immunoreactivity (*e.g.* Western blot), and enzymatic or biological activity.

In this Annex, a review of current knowledge and use of these methodologies is presented, as well as other potentially valuable tools. In particular, cell-based assays that employ either cells isolated from human or animal tissues or propagated from immortal cell lines appear promising.

4.2. Resistance of proteins to *in vitro* digestion by proteases

4.2.1. Background

Two approaches have been taken to studying the digestability of proteins in relation to allergenic potential. The first uses conditions that are often far from physiological and give a biochemical measure of a protein's overall physiocochemical stability (see Annex 2.3.1). One example of this is the *in vitro* digestibility test proposed by Astwood and co-workers (1996) as a method that could be used to predict likely allergenic potential of a newly expressed protein based on its resistance towards degradation by pepsin. The rationale underlying the use of pepsin resistance tests performed under standardized conditions, as proposed by FAO/WHO (2001) and Codex Alimentarius (2003), is that for some known food allergens, there appeared to be a correlation, even if no direct causal relationship, between their resistance to pepsin and their allergenic properties. A second approach seeks to discover the role that simulated, physiologically relevant digestion plays in the mechanisms of oral sensitization. Such studies characterize the repertoire of digestion products to which the gut mucosal immune system is exposed.

4.2.2. Pepsin resistance test

As mentioned above, incubations of transgenic proteins in a solution containing the proteolytic enzyme pepsin, which occurs naturally in the stomach of man and animals, are commonly used to test the resistance of these proteins against degradation. Usually, samples from the incubation are taken after different time intervals and analyzed for the integrity of the test protein. The rationale underlying the pepsin resistance test is that for some known food allergens, there appears to be a correlation, but no direct causal relationship, between their resistance to pepsin and their allergenic properties. The outcomes of the pepsin resistance tests, rather than seeking to model physiological processes, can be interpreted as a biological measure of protein stability. This is because aspartyl proteases, such as pepsin, require a certain degree of flexibility in their substrates as they act on six to eight residue sections of a protein substrate, which must lie across their active site in an extended conformation. Pepsinolysis tests employ conditions which are far from physiological (Astwood et al., 1996) and are based on a standard "simulated gastric fluid" employed for pre-clinical testing of pharmaceuticals, such as described by the US Pharmacopeia (1995). The aim of such studies is to measure the rate of dissolution of tablets and other solid forms containing orally administered pharmaceuticals. The aim is to measure the dissolution rate, which can be compared to the stomach emptying rate, providing an indicator of bioavailability (Galia et al., 1998). These assays can be important for testing the "bioequivalence" of pharmaceutical preparations. Such assays can be performed in special apparatus filled with fluids such as simulated gastric fluid. These apparatus contain devices, such as paddles or moving concentrated rings that mimic the physical forces exerted upon these solid forms. The ability of this test to distinguish between allergenic and non-allergenic proteins was initially described by Astwood and co-workers (1996) and whilst not completely confirmed by subsequent studies (Fu, 2002; Fu et al., 2002), it is still considered to have some utility when used in integrative risk assessment (EFSA, 2006).

Besides "simulated gastric fluid", supplementary models that are used for testing degradation of potentially allergenic proteins include "simulated intestinal fluid" and isolated ruminal fluid. Sequential treatments with the stomach protease pepsin and the duodenal proteases trypsin and chymoptrypsin may be applied, such as it has been done by Mouécoucou *et al*. (2004). Such models do also have applications outside the field of allergenicity research. For example, sequential incubations with ruminal fluid and pepsin solutions are widely used as an *in vitro* model for the digestibility of dry matter and organic matter of animal feeds (Tilley and Terry, 1963).

Such *in vitro* models have been employed in allergenicity testing in a limited manner, focusing on purified target transgenic proteins and known allergens, incubated with the proteases at a gross excess of enzyme to target protein compared with those that might be encountered *in vivo*. Thus, many protocols have employed pepsin: substrate ratios in the range 1/5 – 1/10 (Astwood et al., 1996; Fu et al., 2002). Such ratios may be considered far in excess of those likely to be found in the stomach. Pepsin secretion has been estimated between 20 – 30 kUnits of enzyme activity / 24h at 37ºC in adults (Documenta Geigy, 1973). A typical adult dietary intake of protein around 75g / 24h gives an indication that approximately 1 unit pepsin is secreted for every 3 mg of protein consumed. This compares with approximately 1 unit pepsin/µg protein used in the pepsin resistance assays. Usually, the pepsin resistance assays monitor the integrity of the test protein during the time period of digestion using SDS-PAGE (Thomas et al., 2004), Western blotting, or, less commonly, enzymatic or other indicators of biological activity.

4.2.3. Conduct of pepsin resistance tests

The report of the expert consultation convened by FAO/WHO (2001) on potential allergenicity of transgenic foods provided detailed directions for the conduct of the pepsin resistance test. These details pertained to, for example, the conditions of protein incubation in pepsin solution (e.g. pH 2) and subsequent electrophoresis and staining or blotting of protein bands in the electrophoresis gel. Nonetheless, the guidelines of the Codex Alimentarius Commission (2003) on the *in vitro* digestibility test employing proteolytic enzyme such as pepsin were of a more general nature and mentioned the need for consistent and well validated protocols. Various studies have sought to ameliorate current

practice by either standardization or incorporation of additional features. These include studies focusing on the pepsin e.g. pepsin: protein ratio, pH of incubation mixture, purity of pepsin etc. For example, ILSI Health and Environmental Science Institute has undertaken a ring test, in which allergenic and non-allergenic proteins were submitted to digestion in simulated gastric fluid at two pH values, i.e. pH 1.2 and 2.0, and at a pepsin: protein ratio of 10 units per microgram of test protein, corresponding to a pepsin : protein weight ratio of 3/1 (Thomas et al., 2004). Samples taken in time series up to 60 minutes after the incubation integrity were analyzed by SDS-PAGE. An agreement was obtained between laboratories regarding the time before disappearance of whole proteins, in particular at pH 1.2. Various conditions that varied between laboratories, such as type of electrophoresis gel and fixation technique, influenced the quality of the results, such as the detection of peptide fragments (Thomas et al., 2004). Furthermore pepsin specificity, as determined using model peptide substrates, is known to change as a function of pH (Cornish-Bowden and Knowles, 1969).

Two other studies have been carried out on the kinetics of the degradation of transgenic Cry proteins from *Bacillus thuringiensis* and two labeled model proteins (Herman et al., 2005; Herman et al., 2003). Their results show that, while experimental conditions and velocities of degradation may differ between separate experiments, the kinetics follow first-order kinetic behavior. In addition, half-lives of these proteins, *e.g.* the time point were 50% has been degraded, can serve as a good parameter for comparison between proteins and results from different experiments.

4.2.4. Models for gastrointestinal digestion of allergenic proteins

Detailed features of stomach function have been revealed recently by modern imaging and computational techniques with respect to the transport of fluids and solids within the stomach under influence of gastrointestinal muscle contractions and movement of solids within the stomach. Calculations thus show that with the intake of foods that have a high content of fluid, the passage time for a dissolved compound to the duodenum can be as short as 10 minutes due to the presence of a fluid "stomach road" within the mixed stomach contents, whilst for solid particles, the passage can be in the order of hours (Pal et al., 2007).

Technological innovations have allowed for the continuous non-invasive monitoring of gastric pH through the use of capsules bound to the gastric wall with nylon suture threads and containing sensitive pH meters and radio transmitters logging the measured data to external receivers. This has been used to monitor the intra-gastric pH in non-human primates and it was found that whilst the pH in the fasted stomach is indeed around pH 2, this rises to a peak above pH 4 (median around pH 5) within 30 minutes after a meal and gradually returns within several hours to the fasted state pH (Chen et al., 2008). This pattern is similar to that previously observed in humans. At present, we have little or no information on the patterns in infants and elderly people with probably less acidic conditions in the fasted state. Such fluctuations and differences in intragastric pH are especially important given the pH-dependent nature of pepsinolysis (Christensen, 1955) and can affect digestion of allergens such as those from kiwi fruit (Lucas et al., 2008).

Various recent publications indicate that proteins that are commonly degraded in the stomach, may sustain passage of the stomach and become allergenic in patients that use medicines that impair the action of pepsin, such as by increasing stomach pH, *e.g.* during medication against peptic ulcers. For example, intra-gastric pH is raised above 2.5 in peptic ulcer patients on acid-suppression medication and newborns and may reduce or stop the digestion of proteins by pepsin (Untersmayr and Jensen-Jarolim, 2008; Untersmayr et al., 2005; Untersmayr et al., 2007; Yoshino et al., 2004). This phenomenon in newborns is accounted for by the fact that their stomachs are not completely functional yet, as has also been discussed in section 2.6, which provides a possible explanation for increased occurrence of allergies in children that disappear in later life stages. Conversely, this may also apply to aged people with impaired stomach function due to ageing (Untersmayr et al., 2008).

A fraction of the proteins and peptide fragments that sustain during digestion can subsequently be absorbed from the intestine, as has been demonstrated by the presence of small amounts of orally ingested stable proteins in the serum of human volunteers and experimental animals (Untersmayr et al., 2007; Yamada et al., 2006). A number of factors can influence the uptake of proteins and peptides from the intestine, such as food constituents (surfactants, alcohol), physiological stress and intestinal diseases (Thomas et al., 2007).

The interaction of a given protein present within consumed foods with the host's immune system not only depends on the degradation of the protein during passage but also upon the uptake from the gastrointestinal tract. Various mechanisms exist by which proteins and other antigens are taken up from the intestines and subsequently processed and exposed. These mechanisms include the uptake by M cells in Peyer patches, dendritic cells in the epithelial cell layer with protrusions in contact with the intestinal content, and uptake through endocytosis by epithelial cells (reviewed by Chehade and Mayer, 2005). Despite the fact that many proteins are degraded to short peptides by the action of intestinal exo- and endo-proteases before uptake and metabolism, the uptake of intact forms of specific proteins from the intestinal tract into body fluids and tissues has been observed, e.g. bovine lactoferrin by mice as reported by Fischer et al., (2007). The intestinal uptake of intact proteins is probably mediated by binding of these proteins to receptors on intestinal cells, such as M cells (Fischer et al., 2007). Also endocytosis of intact proteins by epithelial cells can be mediated by receptor-binding, such as has been observed for soybean ferritin on the apical membrane of human epithelial Caco-2 cells *in vitro* (San Martin et al., 2008). The relationship between stability to simulated digestion and uptake *in vivo* in animal models, has been demonstrated for the α-amylase inhibitor allergens from rice (Yamada et al., 2006).

The considerations above highlight the need to consider the stability of protein fragments formed under conditions of digestion besides the stability of the intact protein. Of particular interest are peptides of which the size would be sufficient to allow to contain at least two epitopes for binding by multiple IgE-antibodies and their cross-linking to receptors on the surface of mast cells, eventually leading to release of histamine and other elicitors of allergic reactions. For example, Honma and co-workers (1996) have observed that 20-mer peptides derived from the egg allergen ovalbumin are still able to elicit histamine release from basophils. Another possibility is that peptides that are too small to be allergenic by themselves may associate to form aggregates of sufficient size to trigger an allergic reaction. It has been well documented in the literature pertaining to preparation of hydrolysates of many protein preparations, including food ingredients such as whey, casein or gluten hydrolysates, that the resulting peptides, whilst often of low molecular weight, are able to assemble into much larger aggregates. That such aggregates can form following gastrointestinal proteolysis is being observed, with, for example, large aggregates of casein peptides forming following pepsinolysis (Qi et al., 2007). The formation of such aggregates may explain why even readily digested allergens, such as Ara h 1, can retain their capacity to elicit histamine release after extensive digestion (Eiwegger et al., 2006).

4.2.4.1. In vitro models for gastrointestinal digestion

In order to sensitize an individual via the gastrointestinal tract, an allergen must have properties which preserve its structure from degradation (such as resistance to low pH, bile salts and proteolysis), thus allowing enough allergen to survive in a sufficiently intact form to be taken up by the gut and sensitize the mucosal immune system (Mills et al., 2004; Taylor and Hefle, 2001). Studies investigating how digestion affects the integrity and immunological activity of allergens in this context use more physiologically relevant models of digestion. These models need to take into account a range of other factors which affect the gastro-intestinal passage of proteins besides proteolysis. A number of factors can namely influence the uptake of proteins and peptides from the intestine, such as food constituents (surfactants, alcohol) and physiological stress (Thomas et al., 2007). Factors such as intra-gastric pH also need to be taken into account. In order to investigate the role of stability to gastroduodenal digestion on allergenic potential, model systems which mimic physiological conditions have been developed using appropriate levels of proteases and including biosurfactants such as vesicular

phosphatiduyl choline secreted by the gastric mucosa and bile salts. This system has been used to study the stability to digestion of a number of food allergens including the 2S albumin allergen from Brazil nut and sesame (Moreno et al., 2005b; Moreno et al., 2005c), the LTP allergen from grape (Vassilopoulou et al., 2006), peanut Ara h 1 (Eiwegger et al., 2006), the allergenic thaumatin-like protein from kiwi fruit (Bublin et al., 2008) and the milk allergens α-lactalbumin, β-lactoglobulin and β-casein (Macierzanka et al., 2009; Moreno et al., 2005a). This model system has undergone a multi-laboratory trial through the FP VI EU funded EuroPrevall project. It is noteworthy that the fate of a protein during an *in vitro* digestibility test would likely be different if the test protein is present as a purified protein in solution in a buffer or included in a complex food matrix.

The gastrointestinal tract also involves physical mixing of foods with progressive addition of digestive secretions of which several physical/mechanical modeling approaches have been described (Mitea et al., 2008a; Wickham and Faulks, 2007). Such "Dynamic models" may or may not remove the products of digestion but have the advantage of mimicking the physical processing and temporal changes that actually occur in the gut lumen *in vivo*. This is particularly useful where the physical properties of the digested food changes over time, with regards for example viscosity and particle size and can take account of the formation of unstirred layers and the formation of colloidal phases in the digesta.

There are currently two types of dynamic model available. The TNO Gastro-Intestinal Model (TIM) model takes into account the mixing of gastrointestinal contents under influence of peristaltic muscle contractions and is composed of various sequentially linked compartments representing the stomach, duodenum, jejunum, and ileum, lined with membranes. Enzyme solutions representing the *in vivo* gastrointestinal fluids are added to the pertinent compartments. The uptake from the contents of this artificial gut is measured by their presence of diffusates through the membranes of each compartment. A food sample is introduced and subsequently samples are taken at various stages of the model at specific time points (Mitea et al., 2008a). It is cautioned, though, that this model focuses on passive diffusion and that active uptake by intestinal tissues is not accounted for in this model (Yoo and Chen, 2006).

A second model is built on a modular design of three stages (Wickham and Faulks, 2007). The first part simulates the main body of the stomach, mimicking the mixing dynamics, diffusion profiles of both acid and enzymes and emptying cycles measured within the main body of the human stomach. This is followed by a unique emptying routine into a second module simulating the antrum (the lower part of the stomach). Here the digesta are subjected to high shear (as measured using magnetic resonance imaging), forcing mechanical breakdown of the food structure. The final stage of the model provides a simulation of the small intestine, with integrated intestinal mixing dynamics and diffusion with the addition of bicarbonate, phospholipids, bile, and digestive enzymes simulating the complex environment of the small intestine. Whilst not applicable to studies using purified allergens, such physical models are highly relevant to the study of allergens within the food matrix.

4.2.5. Interpretation of in vitro protein resistance test outcomes

A key aspect in the interpretation of the results of digestion studies is whether they relate to the pepsin resistance test or investigations into the role of physiological gastrointestinal digestion and the allergenicity of foods. With regard to the former, the resistance of a food protein to degradation by proteases has been proposed as an indicator for enhanced likelihood of allergenicity (Astwood et al., 1996). However, subsequent studies have indicated that the relationship between resistance to pepsin and allergenic potential is not clear-cut with some notable allergens being susceptible to digestion under certain conditions (reviewed by Bannon et al., 2003; Fu et al., 2002), whilst several food proteins not known to be allergens show resistance to degradation (Herman et al., 2007). However, the lack of a standardised protocol regarding differences in the pH of the assay, enzymes to protein ratios, target protein purity and method of analysis may cause the variability in these digestibility

studies and makes interpretation of the results from digestion studies difficult to interpret (Thomas et al., 2004).

A much larger body of work relates to studies seeking to define the relationship between gastrointestinal digestion and the mechanisms of allergy regarding both sensitization and elicitation potential. Using more physiologically-relevant digestion protocols, many researchers have shown that peptide fragments derived from the degradation of allergens may still be reactive with IgE from patients' sera. This has been demonstrated, for example, with hen egg's ovomucoid exposed to simulated gastric fluid (Takagi et al., 2005) and grape-derived lipid transfer protein sequentially exposed to simulated gastric and intestinal fluids (Vassilopoulou et al., 2006). Other studies indicate that known IgE epitopes can sustain digestion in the form of such large fragments, such as Ber e 1, the allergenic 2S albumin from Brazil nut. A major degradation product of Ber e 1 is composed of three peptide fragments bound by disulphide bridges (Moreno et al., 2005c). However, there are also studies which indicate that some allergens, such as the major peanut allergen Ara h 1 and the avocado allergen Prs a 1, retain their allergenic activity with regards elicitation potential even after extensive degradation (Diaz-Perales et al., 2003; Eiwegger et al., 2006). Allergens associated with Oral Allergy Syndrome, such as the apple allergen Mal d 1, are generally liable to gastroduodenal digestion and whilst the IgE reactivity of such proteins is destroyed, it appears that T-cell reactive peptides remain (Schimek et al., 2005). The differential resistance of the Bet-v-1- and lipid-transfer-protein- (LTP) types of food allergens has been well documented in foods such as apple and hazelnut (Akkerdaas et al., 2005) and is now being extended to fruits such as kiwi (Lucas et al., 2008), possibly relating to the differential susceptibility of kiwi Bet v 1 homologues and allergens such as the thaumatin-like proteins and actinidin, which are highly resistant to digestion (Bublin et al., 2008).

Such differences have led some, such as Jiang and co-workers (2007), to propose that it is possible to distinguish between food allergens that sensitize through the oral route and those that do not sensitize through the oral route, as is the case for pollen-food and latex-fruit cross-reactive allergens. These authors conclude that most of the allergens with a comparatively large size of predicted peptide fragments after digestion belong to the allergens that sensitize through the oral route. Interestingly, Lucas and co-workers (2008) found that kiwi-allergic patients that show either Oral Allergy Syndrome or systemic allergies also react differently, i.e. the first with pepsin-sensitive proteins and the latter with resistant proteins. Others, such as Moreno (2007), suggest that digestibility should be considered in conjunction with the abundance of the protein in a food, with a higher abundance indicating increased likelihood that some of the protein will escape from intestinal degradation.

For the interpretation of the results, it should also be considered that various experimental conditions have their influence on the resistance of proteins towards pepsin:

- pH: similar to the *in vivo* conditions in peptic ulcer patients on medication and newborns, raising the pH of simulated gastric fluid above pH 2.5 may reduce or nullify the digestion of proteins (Lucas et al., 2008; Untersmayr et al., 2005; Yoshino et al., 2004).
- The ratio between pepsin and protein can substantially affect the resistance of the intact protein and derived fragments. Takagi et al. (2005), for example, incubated hen egg white ovomucoid with pepsin at 10, 1 and 0.1 units of pepsin per microgram of ovomucoid. These authors observed that the stability of part of the intact protein and derived fragments, including IgE-binding ones, increased substantially to more than 30 minutes at the lower incubation ratios of 1 and 0.1 units of pepsin per microgram of ovomucoid (Takagi et al., 2005).
- Food processing and the food matrix will both affect the susceptibility of proteins to digestion, their release and presentation to the immune system and hence their allergenic potential. Pre-heating proteins, as a model for food processing prior to consumption, may also influence digestibility (Takagi et al., 2003). In addition, the conditions of heating may also influence the presence of allergenic proteins in the consumed end product, such as it has been observed in boiled versus roasted peanut with less allergens in the first caused by extraction into the boiling solution (Mondoulet et al., 2005). The matrix can influences digestibility. For

example, when presented in an emulsified form, large polypeptide fragments of casein, which are normally rapidly broken down to small peptides, can resist gastric digestion (Macierzanka et al., 2009). Other food components, such as polysaccharides may also modulate digestion (reviewed by Moreno et al. (2007); see also Annex 2.7.4 on food processing and the matrix).

Codex Alimentarius and EFSA guidance acknowledge that digestibility cannot be completely predictive. As discussed within this Annex, many food allergens that sensitize through the oral route display stability to digestive conditions as demonstrated by the intactness of the protein or derived peptide fragments under these conditions. This stability to in-vitro digestibility should therefore still be considered a risk factor but not in isolation, *i.e.* the outcomes of the digestibility assays should be regarded in conjunction with the results of other assays and the other properties of the protein under consideration.

4.3. IgE binding tests

Various tests for the binding of IgE-containing sera from allergy patients to a test protein are available, and their application for the assessment of potential allergenicity of proteins has been reviewed by Goodman and Leach (2004) and by Goodman (2008). This in some cases may be a variation on the clinically exerted routine assays of testing IgE binding to standardized allergens as a tool for diagnosis of allergies towards these specific allergens. The use of binding tests in which patients' serum is incubated with allergens and the IgE antibodies bound by allergen subsequently measured is provided by various suppliers as an automated assay. These tests may also include positive controls consisting of well-characterized, IgE-containing sera from allergy patients. Less-characterized sera used, for example, in experimental research may also show weak reactions due to the presence of low-affinity antibodies and non-specific binding. The results of IgE-binding assays provide valuable indications for the potential occurrence and intensity of sensitization to allergens. However, the presence of IgE in some cases may not concur with clinical reactions to allergenic foods (van Ree et al., 2006). In addition, sensitive reactions to foods may occur through non-IgE-mediated mechanisms.

IgE-binding assays require the availability of sera from multiple patients, given the variability in specificity and affinity of IgE antibodies between patients allergic to the same allergenic food. Specificity towards specific allergenic proteins is important if, for example, the protein to be tested has shown similarity to a specific allergenic protein in the bioinformatics-supported comparisons with allergens. In cases of rare allergies, the number of sera that are available may be less than optimal, a problem which is also encountered by manufacturers of commercial IgE-detection kits (Hamilton and Franklin Adkinson, 2004). In fact, the OECD's International Co-ordination Group for Biotechnology performed a survey among its members regarding the availability of centralized repositories or stocks of sera that are medically documented and suitable for testing the allergenicity of foods in 1997 (OECD, 2002). It turned out that a number of member states already had such sera banks in place, while some others either had non-centralized points where such sera were kept (*e.g.* companies), or none at all. For the selection of sera, the clinical data of the patients are important. Patients whose sera are used should have a confirmed history of allergic reactions to food allergens (van Ree et al., 2006). In addition, care should be taken to avoid non-specific binding to the sorbent on which the allergen is coupled, for example by diluting sera containing high levels of IgE.

Based on the abovementioned considerations, the characteristics of the sera should be checked with regard to IgE- binding to specific allergenic proteins (e.g. by ELISA and/or immunoblotting), the allergic reactions in the patient (case history and recent confirmation clinical tests, including skin prick test and double-blind placebo-controlled food challenge), and a sufficiently high IgE titer (yet not derived from patients showing broad-spectrum reactivity, such as atopic dermatitis and lupus patients) (Ballmer-Weber and Fernandez-Rivas, 2008; Goodman, 2008).

Guidance from FAO/WHO Expert Consultation (2001), the Codex Alimentarius Commission (2003) and the EFSA GMO Panel (EFSA, 2006) discern **"specific" and "targeted" serum- screening.**

"Specific" pertains to serum IgE antibodies that are specifically directed towards an allergen. This allergen may be the newly expressed protein or an allergen whose structure is sufficiently similar to this protein to provoke a cross reaction with IgE antibodies. It also may be the source of the transgene used in the genetic modification. "Targeted" serum- screening pertains to the use of sera whose IgE are directed towards allergens that are broadly related to the source of the transgenic protein. Codex alimentarius recognizes, however, that "targeted" screening is still in preliminary phase of development.

One example of an IgE-binding assay is the **Radio Allergosorbent Assay (RAST)**, in which an allergen has been absorbed onto a paper disc. This disc is incubated with sera, after which the non-bound sera is washed off and the bound IgE antibodies detected by subsequent binding with ^{125}I-labeled anti-IgE antibodies. The amount of ^{125}I on the disc, which is an indirect measure for IgE bound to the allergen is measured by its scintillation. Automated versions of this type of assay are currently on the market, which allow for quantitative analysis and which employ standardized allergens absorbed to the discs, and in several cases the paper and the radioactive label substituted by alternatives as to finally measure an absorbance or fluorescence.

In the case of the testing of a purified novel protein, it can be envisioned that this protein is absorbed to the disc and exposed to sera from allergic patients to determine whether it is recognized and bound by these sera. In case any possible changes in the crop's own allergenicity due to genetic modification are tested, both discs with whole protein extract from the GM crop and its non-transgenic comparator are exposed to the same sera in order to determine whether a change in the reactivity towards the sera has occurred. However the precision of the quantitative determination allowed by the test may not be sufficient to evidence differences between the two proteins/extracts. If two proteins or extracts are to be compared, an "inhibition assay" may be thus performed in which the antisera are pre-incubated with increasing concentrations of the comparator protein or extract, after which exposure of the allergosorbent disc is to take place. In case that the comparators compete with the allergosorbent for binding by IgE, the first incubation will decrease the amount of unbound IgE antibodies that are still available for binding to the allergosorbent in the second incubation, which is measured as diminished binding to the disc. Comparison of the inhibition curve plotted with increasing concentration of competitors, i.e. the protein or extract from the GM and the non-GM crops, provide useful information on their respective IgE binding capacity.

Variants to the RAST assay include, among others, the **Enzyme Linked Immunosorbent Assay (ELISA)**, which largely follows the same principle as the allergosorbent assay, albeit that it involves the use of plastic tubes or titre plates, colored or fluorescent substrates, and dilution series. Annex 5 provides a more detailed treatise of the ELISA technique as it can also be applied for testing whole extracts of proteins. Moreover, the CAP system may currently be preferred over RAST by various users because of higher sensitivity and lower non-specific binding.

Another method is the use of electrophoresis, followed by immunoblotting with IgE-containing antisera, by which the reactivity with specific protein bands can be discerned. This may be applied to a protein of interest in order to verify whether it is bound by IgE antibodies or not. It may also be applied on a whole protein extract to determine whether any changes in the profiles of IgE-binding proteins have occurred in case the crop that has been genetically modified is allergenic in its own right. A popular technique for protein electrophoresis is SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) for which usually reducing conditions are applied that cause the cleavage of disulfide bonds between proteins or peptides during gel electrophoresis. Several allergens, however, are known to be post-translationally cleaved into peptides that are still held together by disulfide bonds. The detection of the correct whole protein form of such allergens requires the use of non-reducing conditions. In addition, experimental conditions, such as the use of different blocking agents to prevent non-specific binding on the immunoblot, may influence the quality of the results (Goodman and Leach, 2004).

Both for immunoblotting and ELISA, the use of additional control inhibition assays may be warranted in order to verify the specificity of antibody binding. This can be done by inclusion of inhibitors (such as the purified allergen to which the IgE is known to bind) in the incubation solution during the binding of the IgE- antibodies to the protein of interest. This kind of inhibition assays can also be used to further explore cross-reactivity by using the potentially cross-reactive protein as the inhibitor (Goodman, 2008).

Various rapid techniques that may substitute gel electrophoresis and that are amenable to automation have been developed, such as capillary electrophoresis but these are not commonly used yet for the purpose of allergenicity assessment. Capillary electrophoresis (CE) has been used in combination with mass spectrometry (MS) for the analysis of intact proteins since 1989 (Haselberg et al., 2007). For the analysis of allergens it has been used so far by Punzet et al (2006) for the profiling of preparations of recombinant birch allergen Bet v 1A. In that case, the concentration of the recombinant allergen was very high and the sequence was known. It has not been used to detect and characterize allergens in food.

Protein fragments, *i.e.* peptides, may also be tested for IgE-binding such as for identification of epitopes. Usually overlapping fragments of the sequence of the protein of interest are synthesized, for example as fusion peptides with larger proteins or covalently linked to beads. Binding of the single peptides by IgE-containing antisera is tested by incubation in wells of microtitre plates or dot-blots on membranes, followed by detection of bound complexes, such as by colorimetry. This technique may be particularly suited to find **linear epitopes** of a number of contiguous amino acids.

A similar technique to detect non-linear conformation technique is described by Untersmayr et al. (2006). In this case, random peptides were generated as part of recombinant bacteriophage proteins with the "biopanning" technique. Phages that produced proteins that were recognized by IgE directed against the cod allergen parvalbumin, *i.e.* Gad c 1, were isolated and further purified. The peptide sequences that had been inserted into these phages were then identified. Using bioinformatics, these sequences were aligned with amino acid residues, particularly the charged ones that occur on the surface of the known three-dimensional structure of parvalbumin. This combination of IgE-binding with bioinformatics, which is discussed elsewhere in this document, thus enabled identification of non-conformational epitopes. This study also shows that linear sequences can act as "**mimotopes**" of non-linear counterparts in antibody-binding.

Detection of potential cross-reactivity with known allergens by using sera from sensitized animals

The problem of using patient's sera is that the quantity is limited and the variation in reactivity and recognition of allergens between patien sera is high. The antibodies of different patients might recognize different epitopes in an allergen. Furthermore, the epitopes of allergens can be linear but also conformational. Processing of an allergen-containing product or the matrix in which the allergen is present might diminish the allergenicity for one patient, but not for the other. Instead of using sera from patients which are very variable and limited in quantity for the detection of allergens in GMOs, a prescreen of detecting putative allergens could be by using antibodies raised in mice or in rabbits. A model of sensitization to purified food allergen such as b-lactoglobulin or to whole foods (cow's milk and peanut) was developed using a T helper Th2-biaised strain of mouse, i.e. the BALB/c mouse and showed that intra-peritoneal (i.p.) and intra-gastric (i.g.) sensitizations in presence of adjuvant induced the production of IgE antibodies specific to the same proteins, and even to similar epitopes on the different proteins, as human IgE from allergic patients (Adel-Patient et al., 2005).
By over-exposure of mice against the native known allergen as well as the linear (denatured) allergen it is possible to raise specific monoclonal antibodies with known and reproducible charcteristics of specificity and affinity wheras polyclonal antibodies characteristics are multiple and variable. The mice antibodies could be used as a detection for putative allergens, either by immunoblot screening after 1D or 2D protein gel electrophoresis, or by immuno-capturing or immuno-purification. They might recognize a different epitope than the 'real' epitope, and therefore an in depth analysis of the

amino acid sequence of the epitope of the putative allergen is necessary. This could be performed by mass spectrometry sequencing (MSMS).
Small amino acid sequence variation in an epitope of an allergen can make the difference in being allergenic or not (Gao et al., 2008; Schenk et al., 2009; Vader et al., 2002), therefore it will be necessary to characterize the allergen, after the primary screening, in more detail
The method of raising mAbs in mice against immuno-toxic epitopes has been performed for wheat gluten epitopes that can stimulate binding and proliferation of T-cells in celiac disease patients (Spaenij-Dekking et al., 2004). Since gluten intolerance does not develop via IgE, but via T-cell mediated immune response, human sera of celiac disease patients could not be used to detect immuno-reactive epitopes in an immunoblot screening after 1D or 2D protein gel electrophoresis. Although the T-cell epitopes are known and used in raising the mAbs in mice, differences may exist in the affinity and specificity of the mice antibodies compared to the human T-cell lines in relation to the specific amino acid sequence of the gluten epitopes (Mitea et al., 2008b).

4.4. Cell-based assays

4.4.1. Basophil assays

Diagnostic relevance of basophil activity
As described in section 1.2, basophils are involved with the elicitation of allergic reactions by releasing mediators, such as histamine, after contact with IgE-bound allergens. These cells can be used *in vitro* after collection of blood specimens containing these cells from allergic patients. Basophils carry a high-affinity receptor for IgE antibodies. Dimerisation of such receptors at cell surface through the binding of two IgE antibodies to 2 distincts epitopes on a single allergen molecule or to 2 identical epitopes on an allergen with repetitive moieties or present in a polymerized form transduces a signal of activation to the cell. This results in the liberation of preformed mediators, including histamine, heparin and various enzymes such as tryptase, and in cell activation leading to *de novo* production of mediators including leucotrienes.

The system may be sensitive, but suffers a number of drawbacks, among which:

- the number of circulating basophils is low, both in man and in animal models;
- the extent of degranulation in the presence of a given concentration of an allergen is inversely proportional to the degree of allergen polysensitization, which reduces the likelihood of having 2 IgE of the same specificity in sufficiently close proximity as to dimerize;
- basophils can degranulate in the absence of IgE antibodies. Complement breakdown products such as anaphylatoxins (C3a and primarily C5a) are potent activator of basophils;
- the inherent susceptibility to degranulate can be altered by medications such as morphine and derivatives;
- more recently, receptors of natural immunity, called TOLL-like receptors, have been shown to modulate the reactivity of basophils and mast cells. TOLL-like receptors signal down the cell after ligation of many different ligands, from virusses, bacteria, fungi and endotoxins.
- High variance of outcomes depending on the conditions under which the test is performed, including the choice of individual human serum with which the basophils are coated as well as of the donor basophils themselves (Pedersen et al., 2008).
- Setup of the assay may be demanding, for example in terms of logistics (e.g. sampling and transport of donor cells), laboratory facilities (e.g. skilled and experienced staff), and costs (Kleine-Tebbe et al., 2006).

Attempts to circumvent these difficulties include alteration of the protocol for testing, such as introducing a washing step by which all IgE antibodies are eliminated from cell surface. Cells can then be incubated with any serum that putatively contains IgE antibodies to the relevant allergen (passive sensitization assay).

Basophil degranulation tests, which require a processing of blood samples immediately after collection, may also not be suitable. Several cell lines have been derived, primarily from rat basophil leukemias (RBL). However, the rodent alpha chain of the sFcReI does not bind to human epsilon chain. This has prompted researchers to transfect RBLs with the different chains of the human receptor. An *in vitro* functional test using a humanised stable mast cell line is of great interest to study the biological activity of purified proteins or to study the effect of processing or digestion on their allergenic potential. RBL SX-38 cells are derived from rat mast cells that have been transfected to stably express the α, β and γ chains of the human high-affinity receptor for IgE, FcεRI, allowing IgE of allergic patient sera to bind on their surface (Blanc et al., 2009; Dibbern et al., 2003; Wiegand et al., 1996).

In the future, transfected RBLs could become the reference cell for basophil activation test, provided technical difficulties inherent to the use of human serum can be resolved.

Assays of basophil activity

Histamine release can be evaluated by radioimmunoassay or ELISA. Histamine is highly susceptible to degradation upon storage, particularly at room temperature, and stabilization of histamine by acylation is a recommended step before carrying out the assay. The determination of tryptase concentrations, i.e. the serine proteinase enzyme occurring in secretory granules of mast cells, is of interest as a kit is commercially available to evaluate very low concentrations of tryptase. In addition, tryptase is much more stable than histamine. The mediator release can also be quantified by the determination of β-hexosaminidase activity.

An alternative to evaluation of mediators such as histamine and tryptase, is the determination of the number and intensity of basophil activation using surface markers. Thus, surface molecules such as CD63 and/or CD203c show much increased expression after basophil activation and this can be accurately measured with a specific antibody and fluorescence sorting. In fact, the CD63 molecule is associated with the membrane of granules which are expelled after cell activation.

Yet, very large differences between individuals are observed, inter alia because the number of receptors for IgE varies as a function of circulating total IgE. This leads to variable transduction and tyrosine kinase phosphorylation and, consequently, variable degrees of degranulation. CD63 is not specific for basophils and contamination of the cell suspension by activated platelets can indeed generate falsely positive results. It is therefore suggested to combine two antibodies, one directed towards CD63 and another towards IgE.

On the whole, the basophil activation/degradation test is commendable in situations in which different allergens or forms of allergens (e.g. natural versus recombinant) have to be compared for their capacity to bind IgE. This pertains in particular to scenarios in which a transgenic protein has been found to cross-react with IgE from sera, and which therefore needs further confirmation of the capacity of the pertinent protein to elicit allergic responses. Scientific literature provides accounts of encouraging results obtained through studies into the potential cross-reactivity with passively sensitized basophils with other allergens. For example, De Leon et al. (2005) observed the activation of basophils coated with anti-peanut IgE with extracts from peanut, walnut, and Brazil nut, measured as CD63 induction by means of flow cytometry. Wallowitz et al. (2007) also showed that basophils sensitized with sera from a patient allergic to both sesame and walnut were activated to recombinant analogues of the 11S globulins from both sesame (rSes I 6) and walnut (rJug r 4) and with whole extracts from both as well, which also had shown cross-reactivity in IgE-binding assay. If basophils were sensitized with sera from a patient allergic to sesame but tolerant to walnut, despite IgE-cross-reactivity to both, the basophil assay only showed activation in response to incubation with sesame extract or rSes I 6, thus demonstrating the potential utility to discern between relevant and irrelevant IgE-binding (Wallowitz et al., 2007). Basophil tests, once their applicability has been established and validated, would make a valuable contribution to testing transgenic proteins for their potential allergenicity.

4.4.2. Other assays under development

The T cell systems discussed below are in a particularly advanced stage of development, as is the current understanding underlying the mechanisms of their activity.

Diagnostic relevance of T cell activity

As described in Annex 1, T cells play a central role in the sensitization phase of the development of allergies besides their role in the effector phase. T cells are key components of the allergic immune response. It is classically considered that Th2 cells are prominent by their capacity to produce IL-4, an essential cytokine in the production of IgE antibodies. However, not all experimental evidence can be explained by Th2 involvement and there is good data to show that Th1 cells could well be a key player. Whatever the case, the situation is likely to change according to the allergen considered and it is therefore safe to design methods by which specific T cells can be detected without a priori conception on their lineage. This section further discusses the distinction between various lineages of T-helper (Th) cells with regard to its applicability for cell-based assays for allergenicity, whilst the Th17 subset, including both effector and regulatory types of Th17, is not further elaborated here given that its existence has only recently been discovered (see Annex 1.3 on mechanisms).

The development of T helper cells into either Th1 or Th2 categories has been found to depend, among others, on the activity of two specific transcription factors, GATA3 and T-bet1. Transcription factors activate genes by "switching on" their transcription. GATA3 binding to consensus sequences triggers the transcription of canonical cytokines belonging to the Th2 lineage, such as IL-4, IL-5 and IL-13. These cytokines are associated with physiological events including isotype switch to the production of IgE antibodies, migration and maturation of eosinophils and mast cells, inhibition of apoptosis in such cells. The overall result typifies a so-called Th2 response. By contrast, translocation of T-bet drives the transcription of IL-2, IL-12 and IFN-gamma, typical of a Th1 response with production of IgG antibodies and antigen-presenting cell activation. Importantly, T-bet is dominating in the sense that activated T-bet binds to GATA3 and inhibits the function of the latter. Therefore, GATA3 activation and its consequences on immediate hypersensitivity reaction develop only in the absence T-bet activation.

A further degree of complexity arises from the observations that the emergence of an allergic reaction could in fact be due to a lack of suppression. Suppressor or regulatory T cells belong to 2 different categories, although some of their characterstics overlap such categories. Natural regulatory T cells are actively selected in the thymus, while induced regulatory T cells are generated in the periphery upon antigen challenge.

A classical example of induction of regulatory T cells, which can also be useful for the assessment of GMO allergenicity, is the induction of Th3 cells obtained by mainly oral administration of antigen in animal models. Th3 cells produce high levels of TGF-beta, a regulatory cytokine, and various amounts of IL-4 and IL-10. Such cells are found in mesenteric lymph nodes after tolerance induction. It is expected that at least part of the mechanisms by which tolerance to food antigens is established is through the elicitation of such Th3 cells. Although the exact role of TGF-beta in such tolerance is still controversial, conditions required for expanding Th3 cells are being investigated.

Assays of T cell activity

Assays for specific T cells include proliferation tests, phenotypic characterization including cytokine production and single cell analysis.

The capacity to proliferate upon antigen presentation is evaluated using either radioactive or fluorescent markers, which incorporate into dividing nuclei and can be readily measured by gamma counting or fluorescence emission. T cells are obtained from the spleen of mouse immunized by conventional methods with either the transgenic protein or its appropriate controls.

A phenotypic evaluation can be carried out on the same cells. Cell surface markers such as ICOS can be detected using specific antibodies and a fluorescence cell sorter. The production of cytokines can be evaluated by either measuring them in the supernatants using an ELISA, or directly within permeated cells. This can easily be combined to an evaluation of the activation stage of several transcription factors; the latter include mainly GATA3 and Bet1, respectively characteristic of the Th2 and Th1 lineage as described above. Methods for the evaluation of transcription factor activation are routinely available using specific antibodies, Western blotting or immunoprecipitation assays.

The ELISPOT assay is a capture assay in which cytokines produced by activated cells are trapped onto membranes coated with specific anti-cytokine antibodies. This method allows an enumeration of activated T cells and therefore allows a direct comparison between transgenic proteins and their counterparts, *i.e.* positive and negative controls.

Assessing the capacity to expand regulatory T cells or elicit induced regulatory cells is usually performed by functional assays. Yet, specific phenotypic markers are being identified, as for instance surface expression of the IL-7 receptor (CD127). Functional assays are based on the property of regulatory T cells to suppress the activation of bystander T cells, *i.e.* T cells of unrelated specificity but sufficiently close to the regulatory T cells as to be affected either by cytokine production or cell-cell contact.

Evaluation of allergen-specific T cells activity

Peripheral blood mononucleated cells (PBMC) are prepared by gradient density centrifugation and mixed for 5 to 7 days with the allergen under scrutiny. Cells are then washed and cultured for an additional 18 h with tritiated thymidine, followed by further washing and counting of radioactivity. The intensity of radioactivity is proportional to the proliferation of cells. By comparison with a control culture in which no allergen is included, it can be determined whether or not peripheral blood cells contain allergen-reactive T cells.

By measuring the cytokine concentrations in the supernatants of such cultures, it is possible to assign a phenotypic signature to such cells. In particular, the presence of IL-4, IL-5 and IL-13 in such supernatants indicate that activated cells pertain to the Th2 polarized CD4+ T cell subset, which is implicated in the production of IgE antibodies. In contrast, the presence of IFN-gamma indicates that such cells can participate in chronic inflammation through the secretion of various inflammatory mediators.

Polyclonal T cells contained in the PBMC sample can be further purified by limiting dilution until cell lines or even clones are obtained. Such cell lines or clones can be further activated by exposure to allergen presented by autologous antigen-presenting cells. This allows not only to evaluate at single cell level the phenotype of allergen-reactive cells, but also to identify the epitope(s) recognized. To this end, synthetic peptides of ± 20 aminoacids are produced, which encompass the entire allergen sequence with an overlap of ± 5 aminoacids. The activation properties of peptide(s) can then be further refined by the use of mutated peptides including single aminoacid substitution.

Interpretation

As with antibodies, including IgE antibodies, the mere presence of allergen-reactive T cells in peripheral blood does not per se establish a diagnosis of allergy. The potential harmful effect of such T cells can be harnessed by intrinsic and/or extrinsic mechanisms of tolerance, namely anergy or apoptosis occurring in gut lamina propria, or by the local presence of regulatory T cells, respectively.

Large variations in extend of proliferation are expected between individuals, and it is therefore mandatory to include a control with unstimulated cells for each assay, which has to be carried out in triplicates.

4.5. Conclusions and recommendations

Various *in vitro* assays have been considered above, some of which have already become a commonplace in the comprehensive testing of transgenic proteins in the risk assessment of GMO. Examples of this are the *in vitro* digestion of proteins in simulated gastric fluid and the use of IgE binding tests to measure potential cross-reactivity of the new protein with known allergens to which the serum donors are allergic. Other assays, such as the cell-based assays are not yet routinely used for this purpose, but appear to be promising as they can mimic the *in vivo* cascade of sensitization and elicitation, whilst obviating the need for clinical testing.

It is noted that the choice and design of the experiments performed for this purpose depend upon other items considered in the "weight-of-evidence" approach. For example, if bioinformatics studies show that a transgenic protein indicates relevant similarities to a known allergen, this would trigger further serum screening of the protein with sera from patients allergic to the pertinent allergen combined with other *in vitro* assays.

4.5.1. Resistance to in vitro protein degradation

Conclusions

The stability of a protein against the action of protein-degrading enzymes that occur within the gastro-intestinal tract is a feature of various food allergens and is generally considered one of the risk factors for allergenicity.

Not all allergens, such as apple allergen, are known to be stable to digestion and also degraded fragments of proteins can act as allergens. In addition, other factors, such as the abundance of the protein in a food, the way it is contained within the food matrix, and the effect of food processing on the protein may affect the likelihood that a protein will survive passage through the gastrointestinal tract. Moreover, the *in vitro* models used may not reflect the fluctuations in pH and enzyme : protein ratios that occur *in vivo* after consumption of a meal. Combination of the outcomes of the *in vitro* digestibility studies with other information, such as the abundance of the protein within the food and the stability towards food processing may therefore provide useful hints of how the outcomes can be interpreted. In addition, besides the intact protein, also peptide fragments derived from protein degradation still may have the capacity to elicit allergic reactions. The analytical methods to detect the degradation of proteins in *in vitro* digestibility models should therefore allow for the detection of peptide fragments. In polyacrylamide gel electrophoresis (PAGE), for example, the resolution of peptide bands depends on the degree of cross-linking of the gels. Gradient gels are commercially available that allow for a good separation and detection of low-molecular-weight protein fragments. In addition, attention may have to be paid to the choice of stain used for these fragments in the gel in order to ensure sensitivity without artefacts.

In addition, there has apparently been a wide-felt need to standardize the conditions under which *in vitro* digestibility assays are performed so that results from tests carried out in different laboratories can be compared. For example, the half-life of a protein in simulated digestive fluids may provide for a comparable parameter. In addition, the outcomes of the ring trial published by Thomas et al. (2004) has shown that the reproducibility is enhanced at pH 1.2 compared to pH 2.0, such as for the time until disappearance of a protein. The pH value of 1.2, however, does not reflect the pH ranges commonly encountered in stomachs.

Recommendations

- The *in vitro* assay for pepsin resistance of newly expressed proteins should be carried out at various pH values between pH 2.0 and pH 4.0, reflecting the in-vivo fluctuations in gastric pH values. This test could also include the non-physiological pH of 1.2, which is considered to yield comparable results between different laboratories and which is frequently used for this purpose. This enables the analysis of the pH dependence of the pepsin resistance of the intact protein and derived fragments. The time intervals at which samples are taken should reflect the natural passage times of ingested food in the stomach (e.g. in the range of a minute up to

an hour). Also the pepsin activity : protein ratio should be varied, including assay conditions of 10, 1, and 0.1 units of pepsin per microgram of test protein. Both positive controls of proteins known to be stable under these conditions and negative controls of proteins known to be labile, should be included. In order to enhance comparability of the outcomes from assays carried out at different time points or in different laboratories, the half-life of a protein showing first-order degradation kinetics should be reported.

- Besides the intactness of the test protein in the pepsin resistance test, also the occurrence of stable protein fragments should be considered as a risk factor. Therefore should detection methods, such as gel electrophoresis, be insufficient to detect low-molecular-weight fragments of proteins, then alternative methods, like HPLC and MS should be performed.
- If the test protein contains disulphide bridges, the presence of potential larger fragments containing re-associated disulphide-bonded fragments should be verified by isolation and detection under non-reducing conditions.
- Proteins of a particular interest, including those showing stability towards pepsin, can also be checked for their stability towards other gastro-intestinal enzymes and to food processing. In addition, the food- or feed- matrix containing the newly expressed protein and processing are likely to have an impact on the proteins's degradation by proteases (e.g. because of interactions with other constituents and/or structural modifications during processing). The information gained and the value of *in vitro* pepsin resistance test or other digestibility tests would thus be much increased if they were performed in presence of food- or feed- matrix extracts.
- The outcomes of the *in vitro* resistance test should be interpreted with care as they represent model conditions. *In vitro* models used may not reflect the fluctuations in pH and enzyme:protein ratios that occur *in vivo* after consumption of a meal. Combination of the outcomes of the *in vitro* digestibility studies with other factors, such as the abundance of the protein within the food and the stability towards food processing may therefore provide useful hints of how the outcomes can be interpreted.

4.5.2. IgE binding tests

Conclusions

IgE binding tests using allergic human sera may be required in various specific circumstances when potential cross-reactivity of a transgenic protein with known allergens is considered possible. For this purpose, sera from patients allergic to the allergen of interest are needed. It is noted, though, that there is inter-individual variability in the protein-specific IgE responses amongst patients sensitised to the same allergenic food. If sera are pooled prior to carrying out any of these assays, there is a risk that antibodies reacting with less frequently recognized allergens may be diluted to an extent that their cross-reactivity with the test protein can be overlooked. Therefore, the reactivity of individual sera should preferably be checked.

Recommendations

- Serum screening for testing possible IgE-cross reactivity is required if there is any indication of relationship or structure similarity between the newly expressed protein and a known allergen, for example if sequence homology is evidenced in the bioinformatics study.
- Human serum screening tests should be carried out with sera from well characterized individual patients so as to individuate potential responses with IgE antibodies that recognize only minor allergens, which may be diluted if sera pooling is done. Appropriate patients should be selected whose allergy to the source of the transgene or to a known allergen similar to the newly expressed protein has recently been clinically confirmed. Where possible the patients should come from geographic regions where the GM plant is expected to be grown and consumed. The patients should be representative of the allergic subpopulations of the populations that may be exposed to the GM crop through its cultivation within their domicile (respiratory allergy) or consumption (food allergy).

4.5.3. Future methodology, cellular assays

Early inflammatory events leading to food hypersensitivity

The link between allergen exposure and the development of an adaptive immune response characteristic of allergy, including Th2 cell activation and production of allergen-specific IgE antibodies is poorly understood. Deciphering the mechanisms involved in allergen recognition and in the effector arm of inflammation would shed light on the very reason as to why hypersensitivity develops with only some antigens. In fact, many allergens exhibit biological properties unrelated to immunogenicity, such as binding of lectins to glycan chains on the surface of human intestinal cells.

In vitro cultures of intestinal epithelial cells can easily be established. Such cells can be exposed to different extracts of GMOs or their natural counterpart. The consequence of such an exposure can be measured at different levels. These include identification of activated metabolic pathways by kinome analysis, *i.e.* the ensemble of the kinase enzymes that regulate protein activity through protein phosphorylation, and identification of RNA transcripts by full transcriptome evaluation. Whether or not different pathways of activation by GMOs and their natural counterpart would be easily determined, the relative importance of identified activation pathways can be established by using reporter genes for transcription factors and antibodies to phosphorylated kinases.

Selective pathways can be blocked by silencing RNA. This consists in silencing specific genes by addition of RNAs that are complementary to the corresponding mRNA. A particular application of such a technology will be the evaluation of silencing gene coding for receptors of innate immunity such as TLRs and NODs. It is indeed likely that such receptors "sense" allergens and represent a necessary step towards effective immunity.

A further step would be to evaluate intracellular protein formation. This is of importance since many proteins are regulated at the post-transcriptional level. Intracellular staining after membrane permeation and labelling of the intracellular target is detected by Facs analysis. In particular, it can be anticipated that in the very near future the transduction of signals within T cells will become routine in the evaluation of T cell activation. One example of this is the evaluation of the nuclear translocation of NF-kb, a main transcription factor for a number of pro-inflammatory cytokines and anti-apoptotic factors.

Basophil-based assays measuring the activation of these B-cells based on CD63 production have already been validated for the diagnosis of a number of allergies. Their application to the assessment of potential allergenicity of transgenic proteins and genetically modified foods has not been widely reported in scientific literature as yet. Further research is therefore warranted so as to explore the potential applicability to allergenicity assessment of genetically modified products.

In vitro cellular assays are not yet routinely used for the purpose of testing for potential allergenicity of transgenic proteins, yet appear promising. Further research should investigate the possibilities to accommodate predictive cellular assays into the allergenicity assessment of GMOs, such as basophil assays confirming cross-activation of these cells by proteins showing cross-reactivity in IgE-binding assays. For some of these assays, such as basophil assays, usually human materials (e.g. isolated cells, sera) are needed. Also the potential utility of T-cell activation should be further explored.

It is noted that phage libraries of human antibodies are currently available and could be used to create a GMO-specific library of human antibodies for pre-screening. In order not to test only the IgE binding capacity of the newly expressed protein but also its functionality to act as an allergen, the basophil degranulation assay is highly sensitive and specific. This test can be standardized using cell lines transfected with the human FcεRI receptor, such as basophil rat leukemia cells.

Recommendations for further development:

Future work should focus on the developement of cell based tests for assessing the capacity of the newly expressed protein to bind IgE and provoke the degranulation of basophils.

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ANNEX 5. ANALYTICAL AND PROFILING TECHNOLOGY / *IN VITRO* PROTEIN ANALYSIS AND PROTEOMICS METHODS FOR TESTING THE POTENTIAL ALLERGENICITY OF THE WHOLE GM PLANT

5.1. Introduction

The assessment of allergenicity of GM plants should ensure that no unintended effects of the genetic modification would impact on the allergenicity, e.g. will not modify the allergenic protein profile. Any such alterations could lead to undesired over-expression of allergens. Recent comparative studies on the proteome and transcriptome of several GM plants, including tomato, potato, wheat and soybean, have found very few differences between the modified and wild-type forms. Any differences found between the control and transgenic lines are generally within the same range as the differences observed between the control lines grown on different sites and in different years (Baker et al., 2006). In fact, fewer differences were found than between plant varieties. However, each genetic modification should still be examined case by case. For example, Di Carli et al (2009) examined the proteomes of GM plants that were designed to offer antiviral resistance. They compared the protein profiles of two plants expressing different recombinant antibodies with those of their unmodified forms. Tomato was modified to express the antibody scFv(G4) against the cucumber mosaic virus (CMV) coat protein, and *Nicotiana benthamiana* was engineered to express the antibody scFv(B9) against the G1 envelope protein of tomato spotted wilt virus. These viruses both contribute to large crop losses. Seven protein extraction procedures were tested for both plants, to ensure the maximum number of proteins was isolated and detected. The differences in protein profiles between leaf protein extracts from the GM and control plants were highlighted by two-dimensional differential gel electrophoresis, using imaging techniques and a statistical analysis to identify proteins that were differentially expressed. A total of 1818 spots were detected on the tomato gels but only 10 were differentially expressed. Similarly, 8 proteins out of 1989 for *N. benthamiana* were apparently affected by genetic transformation. However, the differences in expression were low with an average ratio of less than 2.4. The proteins were identified by mass spectrometric techniques. The majority were single expression products involved in photosynthesis or defence processes rather than metabolic pathways, so could be caused by "minimal environmental stimuli." Taken together with the low expression ratios, the data led the researchers to conclude that "the proteomic differences observed between GM and control plants are negligible, defined and more likely due to physiological variations" (Di Carli et al., 2009).

The above study used proteomics techniques to analyse differences in protein profiles between GM and control plants, but this Annex will focus on different methods of *in vitro* detection of allergens that might be expressed unintendently due to genetic modification.

When investigating the potential allergenicity of whole plants, special attention needs to be drawn to identify the natural variability in expression levels of identified proteins in wild type plants and to compare the composition of the wild type and the genetically modified organism (Baker et al., 2006; Shewry et al., 2006, see also chapter 4). Furthermore, it needs to be emphasized that posttranslational modifications may alter the overall immunogenicity of proteins. For example, alpha-amylase inhibitor from bean displayed different structures of N-linked glycans when introduced into peas (Prescott et al., 2005). As a consequence enhanced immunogenicity of the GMO protein was observed when testing in an animal model. Although no evidence of altered allergenicity was concluded, the example elucidates that post translational modification needs to be included in allergenic risk assessment.

Allergens are usually only a small fraction of the proteins of a given plant. As an example grasses have only 11 allergenic groups described so far in their pollen and maybe up to 25 allergenic groups in their seeds. A few allergens have been described in their leaves and roots most of them being common to the pollen or the seeds. These numbers are very small as compared to the total number of expressed

and detectable proteins in grasses. So the techniques required to analyze the allergens have to be able to analyze and screen a great number of proteins to be able to detect a very small number of allergens. This detection, as allergens, will be done by a specific recognition with human IgE antibodies.

Two major techniques are available. One introduced in 1972, is the Enzyme Linked ImmunoSorbent Assay (ELISA). ELISA and techniques derived from the same principle are immunoassays for the detection and quantitation of allergens or antibodies in solution. They are able to screen rapidly a great number of different samples in 96 well plates and are able to identify and quantify allergens with the help of specific human IgEs. The second technique combines two dimensional electrophoresis in gel (2DE), introduced in 1975, which has a high capacity to separate proteins, 1000 of them in a gel 10X10 cm, and detection of the allergenic protein spots by immunoblotting, a technique introduced in 1972. Once detected, allergens can be identified using for example MALDI TOF mass spectrometry (see below). An alternative is the use of chromatographic separations using two or even multidimensional liquid chromatography (2D or MD-LC) coupled to mass spectrometry (MS) for identification.
It is noteworthy that the same immunoassays may be used either to detect or confirm the diagnostic of a specific allergy/sensitization in a patient provided that a standard well characterized allergen is used as reference material or to assess the allergenicity/IgE binding capacity of a compound which is related to a known allergen using the serum of relevant and well characterized patients whose allergy to the source (food or protein) is confirmed.
After a short description of these methods, their advantages and limitations will be analysed and alternatives and perspectives will be presented.

5.2. ELISA and derived immunoassays

Derived from the radio-immuno assays, ELISA became very popular by the use of enzyme labelled second antibodies and by its miniaturized format: the microtitration plate, allowing a rapid screening of numerous samples in a reduced amount. Its principle consists in immobilizing one partner of the Antigen-Antibody (Ag-Ab) reaction on a solid surface, the bottom and the walls of a plastic well and let it be recognized and quantitated by the other one in the liquid phase. The classical allergen recognition will be obtained by immobilizing a potential allergen and letting it be recognized by human IgE antibodies, usually called the first antibody, this one being detected and quantified by a second antibody, enzyme labelled. Several improvements to this basic format have been introduced such as the capture antibody or the inhibition assay just to mention two of them.
ELISA has become increasingly popular as most of the critical steps have been automated and recently all of them have been integrated into robots allowing high throughput and routine dosages. High dynamic ranges in sensitivity can be obtained with a limited amount of reagents.
The selectivity of the method is an important point to consider. Only the allergens that can bind the plastic surface will be detectable. These allergens have to be water soluble for their extraction from their original source and for their accurate and reproducible handling and dispensing. But their binding to the polystyrene surface of the plates requires some hydrophobic properties at their surfaces. Several chemical modifications of the microplates are able to correct or adjust the allergen binding to them. Recent treatments of some non water-soluble allergenic extracts of grasses have been made directly in the wells in order to precipitate and bind these allergens on the solid phase. Interfering carbohydrates have been reported: they may act as haptens present on far too many molecules that may be abusively detected as allergens by IgE antibodies to these motives (CCD or cross reactive carbohydrate determinants).
Another aspect of the selectivity of this assay is linked to the immunologic detection. IgE antibodies are present in minute amounts in allergic individual sera, especially after an allergen specific immunotherapy, which induces usually an increase in non-IgE antibodies to the allergens that may interfere with their exact estimation. Even if low affinity antibodies have been reported to be measurable by ELISA their quantification is greatly dependent upon the protocol used such as the detergent concentration, the blocking agent or the duration of incubations. This is particularly true when immunological cross-reacting allergens are tested with a single antibody directed to one of them.

The affinity of this antibody to the immunologically "distant" ones decreases and the assay may not reflect their real amount. Allergens are frequently present under different isoforms or isoallergens. Some allergic individuals do recognize only a few of these isoforms, those who have in common some special epitopes.

The allergen detection by IgE antibodies has been first introduced commercially by the Company Pharmacia from Uppsala in Sweden (now Phadia) soon after the IgE discovery. The first test available was a radio-immunoassay named RAST for Radio Allergo Sorbent Test. The IgE concentration was expressed as kU/L corresponding to approximately 2.4ng/ml. An enzyme immuno assay named ImmunoCap was then gradually replacing the RAST. Its principle is very close to the ELISA but in fact could be named under the generic acronym of ELIFA or Enzyme Linked Immuno Filtration Assay as the solid phase is a sponge on which allergens are covalently immobilized and all the interacting reagents are percolated under pressure through this sponge. The major advantage of this method is the great allergen binding capacity as compared to the conventional ELISA, meaning a very high sensitivity of the method and a great dynamic range: as low as 0.1 kU/L (0.24ng/ml) and up to 100 kU/L of IgE to milk allergens can be detected.

5.2.1. Improvements on ELISA for allergenicity testing

After a long maturation recent improvements are pointing out in the world of analytical techniques applied to immunoassays. The development of the future ELISA for allergen testing will certainly go into three major directions: it will be miniaturized, allowing a great number of allergens to be tested by the same single allergic individual serum and purified allergens will be preferred to crude source mixtures.

The use of purified allergens or of crude allergen sources

Binding a crude allergen source on a solid surface for an immunoassay is not an easy task. The unique chemistry used will fit most of the proteins but will never bind some others. They will be washed out from the surface and ignored during the decisive immuno-detection step. In order to avoid this artefact that may concern major allergens purified natural or recombinant allergens are used for a component resolved diagnostic of allergy. Their rarity or high cost is compensated by minute amounts needed to coat tiny spots on allergen chips. Only a few purified allergens issued from a given allergen source are in fact needed to establish the allergy diagnostic: they will be chosen among the most frequently recognized allergens and be the markers of the allergy to the whole source from which they originated. It is important that the recombinant synthesized allergen has the same conformation and modifications (e.g. glycosylation) as the native allergen (Swoboda et al., 1995).

Miniaturization

Spectrophotometric detections in 50 microM wide windows are since long used in capillary electrophoresis and more recently in micro- and nano-LC (liquid chromatography). Spots regularly arranged on a chip could then be individually probed by light and their absorbance quantified. Since several years DNA arrays of spots are commercially available. Protein or antibody spots in arrays are now produced. For the allergy diagnostic a very simple glass chip, the size of a microscope slide has been proposed, carrying around 80 different allergens in triplicates plus positive and negative controls. A total serum volume of 50 µL is recommended for IgE detection. All the washing and incubation steps are still manual. More elaborate miniaturized equipments are now in development. Most of them keep the array format to allow the screening of several hundred different immobilized proteins that could be allergens by a single antibody source. The use of micro-fluidic reduces greatly the need for serum and allows a completely automated dosage.

Capillary electrophoresis introduced in the 70's has long been the intermediate between the normal slab gels and the miniaturized microchips. An efficient cooling allows very fast electrophoretic migrations. All the steps needed to separate several complex proteic samples can be automated. The recent progress in microfluidics will allow soon several different electrophoresis to be performed one

after the other as is nowadays done in the conventional 2 DE in gel followed by an immunodetection step. So one might have soon the capacity to perform the equivalent of micro 2 DE in gel, fully automated and requiring only nano L of samples to be analyzed and a few µL of sera for their immuno-monitoring.

Microarrays

A recent development in IgE-binding assays is protein microarrays to screen for IgE-binding to multiple allergenic proteins and/or epitopes simultaneously (Harwanegg and Hiller, 2005; Lebrun et al., 2005). At the moment of writing, one company offers a protein microarray containing 79 recombinant proteins from a range of allergens including food-, pollen-, insect-, and animal-dander-allergens.

Microarrays are composed of a slide, such as a microscopic glass slide, onto which tiny spots containing specific probes have been applied. In the case of protein microarrays, these spots contain extracts of allergens, purified allergenic proteins, or fragments of allergenic proteins. Sera containing IgE will be brought onto the slide, incubated, and subsequently washed off, so that only antibodies bound to the proteins in the spots will remain on the slide. Subsequent incubation with fluorescently labeled anti-IgE antibodies and washing off unbound antibodies will allow for the detection of spots with bound IgE as fluorescent spots under a microscope. Also other detection methods besides fluorescence can be envisaged (Harwanegg and Hiller, 2005).

The advantages of using microarrays for IgE-binding assays are that only small quantities of serum are needed, i.e. tens of microliters, which allows for samples to be taken from newborns, for example. In addition, the ability to screen for binding to multiple components of allergens simultaneously allows for "component resolved diagnosis." Through this type of diagnosis, it is possible to detect differences in sensitivities towards different components, also revealing cross-reactivities between different allergens in the same patient (Harwanegg and Hiller, 2005). It can be envisaged that this technology can be extended to the field of GMO safety assessment by inclusion of highly purified newly expressed proteins from GMOs into the microarrays.

5.3. 2D gel electrophoresis and immunoblotting

2DE is the combination of two electrophoretic techniques based on complementary principles, isoelectric focusing (IEF) followed by SDS PAGE, a polyacrylamide gel electrophoresis in a buffer containing a detergent, the sodium dodecylsulfate. IEF separates the protein following their isoelectric point and SDS PAGE separates them following their molecular size relatively to the migration of standard protein markers. As stated in the introduction this technique is able to display in one 10X10 cm gel approximately 1000 proteic spots. In order to have an optimal sensitivity of the technique it is advisable to avoid the analysis of too complex extracts, a limited number of spots will be compensated by a higher concentration of each of them. So, ideally a whole plant will never be studied in one single gel but in a succession of gels each one devoted to a given part or organ of the plant.

Once the proteins of a complex crude extract of a plant are analysed by the 2DE technique the best way to detect the few allergens present among them is to perform a blot or print of this gel on a membrane, usually made of nitrocellulose or a hydrophobic polymer PVDF. The transferred allergens and proteins are immobilized on a solid surface. Their detection is now possible, as by ELISA, by incubation with a relevant antibody solution, usually a human allergic individual serum. It could also be incubated with antibodies raised against the allergen or against a particular epitope of the allergen in animals. The use of monoclonal antibodies with well defined characteristics of specificity and affinity would allow a standardized (pre) screening. When using allergic patient serum, a minimum of 300 µL diluted 10 times in a final volume of 3 ml for a 10 X 10 cm blotting membrane is required whereas 1 D electrophoresis in gel followed by blotting requires as a mean 50 µL. The IgEs specific to the immobilized allergens are then detected by the incubation of the blot with enzyme labelled second antibody (i.e. an anti-human IgE antibody) followed by the addition of the enzyme substrate. The detected allergens are thus characterized by their isoelectric point and their relative mass. Their further

identification requires a peptidic sequence analysis. This is most commonly performed by using mass spectrometry. Each proteic spot identified as an allergen is excised from the gel or its blot, digested by proteolytic enzymes into peptides and the peptide mixture is analysed by mass spectrometry. The mass spectrum of the peptides is then compared to the spectra contained in protein or allergen databanks, or to the translated amino acid sequences present in genomic, cDNA or EST (expressed sequence tags) libraries, allowing the identification of the detected spot.
If a new protein, not yet registered in a protein sequence databases was to be detected in a 2DE in gel followed by an immunoblot one would have to perform its amino acid sequence analysis.
A single 10 X 10 cm blotting membrane may allow the detection and the identification of a few dozen of different spots by using a few mg of a crude allergenic extract.

5.4. Alternative proteomic methods for targeted allergen detection

Once detected, an allergen can be identified not only by its immunochemical and biological properties using tests such as ELISA, immunoblotting or mediator release assays but also by its physiochemical properties. By offering a tool for detection of specific sequences of proteins of interest, novel MS-based technologies are a potential alternative to the 2D-gel electrophoresis approach to multi-dimensional separation of proteins. In regard to the problem of the complexity of the sample, the past two decades have seen a growth of significantly improved mass-spectrometric devices, allowing precise analysis of biomolecules (Seibert et al., 2005). In general, the instruments are made up of three primary components: the source, which produces ions for analysis; the mass analyzer, which identifies the ions based on their mass-to-charge ratios; and the detector, which quantifies the ions resolved by the analyzer. The technique is sensitive to the picomole to femtomole range.

In the targeted approach discussed in this section, the mass spectrometer serves as a selective detector that detects the sequences of specific proteins of interest, whilst in the non-targeted approach discussed in section 7.5, it serves to elucidate the sequence (and hence the identity) of any given protein instead of focusing on a specific sequence belonging to a particular allergen. Tandem mass spectrometry (MS/MS) or MS^n , which is a stepwise fragmentation in which the ion fragments detected in the first step are further fragmented and detected in one or more additional steps, allows the identification of amino acid sequence of peptides using computational methods to analyze the MS fragmentation patterns. Examples of MS/MS techniques through which the sequence identity of a protein can be assured include peptide mass finger printing (PMF) with Q-TOF-MS/MS (Cut of Time Of Flight Mass Spectrometry) or LC-MS/MS (Liquid Chromatography Mass Spectrometry). Moreover, besides the amino acid sequences also other features of proteins can be revealed by MS-based methods. For example, the glycosylation pattern can be revealed by MALDI-MS (Matrix Associated Laser Desorption Ionisation Mass Spectrometry) (Bakker et al., 2006), LC-ESI-MS (Liquid Chromatography Electron Spray Ionisation Mass Spectrometry) or by lectin binding assay (Kronsteiner et al., 2008). A combination of separation based on immunochemical properties and detection based on physiochemical properties can also be used, as is performed in the SELDI-MS (Surface Enhanced Laser Desorption Mass Spectrometry analysis method (Hsieh et al., 2005) or by making a combination of immunoblot screening or immunoprecipitation coupled to mass spectrometry (Bassler et al., 2009; Careri et al., 2007).
In the following sections the focus will be on MS methods used to identify and quantify (isoforms of) allergens.

For the analysis of complex biological matrices generally multidimensional systems are needed based on combinations of different separation and/or detection systems. Today, various separation techniques are available for the qualitative and quantitative analysis of proteins. Frequently used techniques are slab-gel electrophoresis (SGE), liquid chromatography (LC) and capillary electrophoresis (CE) (Issaq et al., 2002). Limitations of SGE are the relatively long and labor-intensive analysis, the necessity of off-line detection, and the lack of precise (and automated) quantitation. LC is advantageous due to its separation power, ease of automation and routine coupling with various detection principles, like mass spectrometry (MS). CE offers attractive features for the analysis of

proteins, as the analysis times can be relatively short and only minute amounts of sample are needed. Furthermore, CE analyses are carried out in fused-silica capillaries under aqueous conditions and in the absence of a stationary phase. This enables the study of proteins without causing conformational changes due to organic modifiers and/or a stationary phase (Haselberg et al., 2007). Kronsteiner et al. (2008) used CZE (capillary zone electrophoresis) to characterize two recombinant products of the birch pollen allergen Bet v1a. However, in the case of putative (new) allergens present in GM crops it will be necessary to characterize the allergen on more than size and charge because small point mutation might render a protein an allergen or not. This information can not be obtained with CE analysis alone. Therefore, amino acid sequence information will be needed for true characterization. Chen et al. (1998) characterized latex allergens by capillary electrophoresis and combined it with N-terminal amino acid sequence analysis of the isolated allergens.
Recently, there has been an increased interest in the development of chip-based analytical systems, as they may increase analysis speed and performance, and reduce cost, weight and size of the instrumentation. Successful efforts have been made to combine microfluidic CE systems with mass spectrometric detection (DeVoe and Lee, 2006; Sung et al., 2005). In the protein analysis field, applications of chip-based CE–MS mainly focus on digests of proteins, but intact protein analysis has also been described in some cases (Haselberg et al., 2007). These chip based analytical systems that couple microfluidic systems with mass spectrometry give more identification of peptides or proteins, but still no sequence information. That could be obtained when using MALDI-MSMS. There are no examples however where this combined chip-based system with MALDI-MSMS is used for allergen detection and identification.

SELDI-TOF MS (surface-enhanced laser desorption/ionization time of flight mass spectrometry), first introduced by Yip and Hutchens (1992), is based on two powerful techniques, chromatography and mass spectrometry. It consists of selective protein/peptide extraction and retention on chromatographic chip arrays and their subsequent analysis using a simple laser desorption/ionization mass spectrometer (Merchant and Weinberger, 2000). The ProteinChip arrays have chemically derivatized surfaces utilizing classical chromatographic separation characteristics such as reversed phase, ion exchange, silica, immobilized metal affinity capture, and preactivated capture. The latter surface allows for covalent attachment of various molecules, such as antibodies, receptors, DNA, small molecules, and ligands. Bio-active proteins/peptides can thus be captured on these surfaces and/or identified through the recognition of their corresponding antibodies.

SELDI-TOF MS technology has thus far been successful in various applications ranging from protein profiling of complex biological mixtures (Issaq et al., 2002) to identification and characterization of biomolecules (Caputo et al., 2003).
SELDI-TOF MS technology could be used for allergen detection in GMO when a set of antibodies raised against the main allergens would be available that can selectively capture allergens present in an extract. The captured proteins can be characterized by mass spectrometry of intact proteins. Again, no sequence information becomes available.
Examples of the SELDI-TOF MS method where allergens are selectively captured by antibodies covalently attached to a protein chip array are not available yet, but could present a promising technique. For isoform identification, sequence information will be needed and MSMS application therefore will be necessary.

Capture of known allergens with magnetic beads coupled to specific antibodies can also be combined with MALDI-TOF/MS. After release of captured allergens tryptic digestion should be performed. This could all be automated or robotized and therefore be made high throughput. A drawback is that it is not quantitative, but it could be made quantitative by spiking with radiolabeled peptides, and that antibodies need to be raised against all known allergens.

Mass spectrometry techniques for identification of (isoforms of) allergens
Several groups used MS/MS analysis to get sequence information for the identification of allergens. The MS/MS technology was combined with immuno-affinity or immunodetection of the allergens in a

complex protein mixture. Helsper et al (2002) used affinity purification of apple allergens in combination with QTOF-MSMS for the identification and semi quantification of different isoform compositions. Also non-allergenic proteins have been identified in such a way. Elvira et al (2008) used immunodetection by Western blot analysis of 2D protein gels of PR proteins in compatible and incompatible viral infections of *Capsicum chinensis* plants to identify PR protein spots. These spots were excised from the membranes and sequenced by MALDI-TOF spectrum and MSMS spectrometry.
In these above mentioned examples, the allergens or proteins are first purified by immuno-affinity or immunodetection in a 2D protein gel, and subsequently characterized by MSMS peptide sequencing. This is a labour intensive method, but small sequence differences in isoforms can be detected. Since different isoforms can have different IgE-reactivity, this sequence information is very valuable.

Multidimensional LC-MSMS combined with immunodetection or immunopurification
Bässler et al. (2009) used for the molecular characterization of tomato seed allergens, a multidimensional protein fractionation strategy and LC-MS/MS. For protein separation they performed two-dimensional chromatography using chromatofocusing (CF) for the first dimension and reversed phase (RP) chromatography in the second dimension. The protein fractions were subjected to SDS-PAGE for further separation. Potential allergens were detected by IgE immunoblotting and analyzed using LC-MS/MS. A legumin- and a vicilin-protein were identified as IgE-cross-reactive tomato seed proteins as new putative allergens showing a significant homology to other previously reported food allergens. EST/Contig sequence alignments combined with tryptic peptide coverage analysis revealed a novel full-length vicilin protein in tomato. This combined method of multidimensional LCMSMS combined with immunodetection is again a targeted and labour-intensive method, but renders sequence information. In this example new putative allergens could be detected in a very complex protein mixture.

Complex matrices commonly affect the sensitivity and selectivity of liquid chromatography-mass spectrometry (LC-MS) analysis. Thus, selective sample enrichment strategies are useful particularly to analyze proteins present in low abundance in samples. A selective immunomagnetic extraction procedure to isolate trace peanut allergen protein Ara h3/4 from breakfast cereals combined with microwave-assisted tryptic digestion and liquid chromatography-electrospray ion-trap tandem mass spectrometry (LC-ESI-IT-MS/MS) measurement was developed by Careri et al. (2007). Using protein A-coated magnetic bead support, anti-Ara h3/4 monoclonal antibodies were used as selective capture molecules. The results obtained by LC-ESI-IT-MS/MS in terms of limit of detection (3 mg peanut/kg matrix) and a significantly reduced matrix effect demonstrated that the Ab-coated magnetic bead was very effective to selectively trap Ara h3/4 protein in breakfast cereals. The magnetic bead-based sample treatment followed by LC-IT-MS/MS method that has been developed can be proposed as very rapid and powerful confirmatory analytical method to verify the reliability of enzyme-linked immunosorbent assay (ELISA) screening methods, since the magnetic bead-LC-IT-MS/MS method combines good sensitivity to the identification capabilities of mass spectrometry (Careri et al., 2007).
The use of a selective immunomagnetic extraction procedure combined with LC-MSMS analysis speeds up the procedure compared to the earlier discussed immunodetection or immunoaffinity extraction procedures. Furthermore, this example shows that allergens can be detected in processed food.
In all the above mentioned examples of proteomics analysis of allergens, an approach was used, in which the allergens are captured or detected using immuno affinity.

5.5. Profiling methods as a non-targeted approach to detect modifications in allergenicity (or antigenicity) induced by the genetic modification

The classical 2D gel electrophoresis for separation of proteins (see section 5.3) is a laborous method, and has to be followed with immunoblot using human sera or monoclonal antibodies raised in sensitized animals in order to detect putative allergens or to identify and characterize alterations

among the expression of several thousands of proteins in a whole GM plant as compared to the non-GM counterpart. This needs to be combined with cutting the immuno-reactive spot out of gel or membrane and MSMS characterization after tryptic digestion of the protein spot for identification.
By using DIGE (Difference in Gel Electrophoresis) the protein pattern of a GMO protein extract can be compared with a control extract in one gel. In this method different fluorescent labels can be used to label proteins and as a consequence different extracts can be run and compared on the same 2D gel. With this method a GM protein extract can be compared to an untransformed organism and an internal standard. All up-regulated protein spots could be identified and cut out of gel for identification. This method visualizes more proteins than only allergens, but is not high troughput and not quantitatively. A drawback of 2D gel electrophoresis analysis is that membrane proteins, highly charged (basic and acidic) proteins and very large proteins are difficult to resolve. Furthermore, low abundance proteins are also difficult to identify in the presence of highly abundant components. Appropiate extraction techniques are therefore needed. For MSMS identification of a protein spot from the 2D gel a relatively high amount of protein is needed (clearly visible spot after CBB, Coomassie Brilliant Blue, staining).

An non-targeted method using mass spectrometry, i.e. LC-QTOF/MSMS, has been successfully used to detect and identify major allergens in processed peanuts by Chassaigne et al. (2007). Before this method can be used in a high throughput way to detect allergens in GM food, different allergen-specific sequence tags need to be identified that can function as markers of the specific allergenic proteins using this method.

Comparitive LC-TOF/MSMS could compare tryptic digests of transgenic extract versus non-transformed organisms and selectively perform MSMS on the differentiating mass peaks. This method is quantitative (America and Cordewener, 2008), but not high throughput. It will give, however, information on all differentiating proteins and peptides in the GM food compared to non-GM.

Isotope or mass tag labelling to assess differential protein expression

Quantifying changes in protein abundance between samples is a key requirement for profiling changes in cell state at a molecular level. One approach uses isotope or mass tag labeling of peptides where two samples to be compared are covalently modified by isotopically distinguishable (*e.g.* 1H *versus* 2H, 12C *versus* 13C, or 14N *versus* 15N) but chemically similar adducts, the samples are proteolyzed, mixed and relative changes in protein abundance determined from ratios of intensities between the differentially labeled peptides using MS. Gygi et al (1999) used an isotope coded affinity tag (ICAT) reagent with specificity toward sulfhydryl groups. As an example, in this case the side chains of cysteinyl residues in a reduced protein in a non-GM sample are derivatized with the isotopically light form of the ICAT reagent. The equivalent residues in the GMO are derivatized with the isotopically heavy reagent. The two samples are combined and enzymatically cleaved to generate peptide fragments, some of which are tagged. The tagged (cysteine-containing) peptides are isolated by avidin affinity chromatography, the isolated peptides separated and analyzed by LC-MS/MS. Both the quantity and sequence identity of the proteins from which the tagged peptides originate are automatically determined by multistage MS using the spectrometer in a dual mode alternating in successive scans between i) measuring relative quantities of peptides eluting from the capillary column and ii) driving sequence information of selected peptides. Peptides are quantified from the relative signal intensities for pairs of peptide ions of identical sequence that are tagged with the isotopically light or heavy forms of the reagent, respectively.

The chemical reaction in the ICAT alkylation can be performed in the presence of urea, sodium dodecyl sulfate (SDS), salts, and other chemicals that do not contain a reactive thiol group. Therefore, proteins are kept in solution with powerful stabilizing agents until they are enzymatically digested. Studies indicate that the accuracy and variability of such ICAT approaches can reliably detect down to an 1.5- fold change in protein abundance over a dynamic range from 10- to 100-fold. Zhou et al

(2002) describe a method for site-specifc stable isotope labeling of cysteinyl peptides in complex mixtures through a solid phase capture and release proicess and concomitant isolation of labeled peptides using LC-MS/MS) to determine their sequence and relative quaktities. The solid phased system appears simpler more efficient and more sentitive than the ICAT systems *per s*e.

Disadvantages include cost of isotopic labeling and equipment for pairwise comparisons between samples, which prevent retrospective comparisons and complicate large studies. Not all proteins contain cysteinyl residues and are therefore missed by using thiol-specific ICAT reagents but ICAT reagents with different specificities make cysteine-free proteins susceptible to analysis by the ICAT method. The ICAT approach provides a broadly applicable means for the quantitative cataloguing and comparison of protein expression in a variety of biological samples. However, to date it has not been widely used for plant tissues.

Label-free protein quantitation methods

Label-free methods for protein quantitation in shotgun datasets offer an alternative approach to stable isotope labeling methods. Peak area intensity and spectral counting methods enable protein ratios significant to 2.5- fold to be determined with high confidence. This is a lower sensitivity compared with isotopic labeling where protein ratios significant to 1.5-fold have been reported (Old et al., 2005 and references therein) but neverless biologically relevant. The ability to achieve this without stable isotope labeling can be advantageous under conditions where metabolic labeling or chemical derivatization is difficult. Old et al (2005) and others have demonstrated that mass spectral peak intensities of peptide ions correlate well with protein abundances in complex samples. Label-free methods, termed spectral counting, compare the number of MS/MS spectra assigned to each protein. An advantage of the spectral counting approach is that relative abundances of different proteins can in principle be measured. Thus, significant correlations have been shown between spectral counts and independent estimates of protein copy number.

Comparitive LC-MSMS has also been used to identify which Betv1 allergenic isoforms are expressed in different birch species. Not only the presence but also the relative abundance could be determined of isoforms with a high and low IgE-reactivity (Schenk et al., 2009). When using MSMS analysis to determine sequence information of the allergens, the availability of DNA sequence information (genomic sequences, cDNA or EST sequences) of the allergens is important to be able to identify the peptides. Since DNA sequencing has been put to a higher level with the next generation sequencers, this type of information is explosively expanding.

5.6. Conclusions and recommendations

Profiling techniques can employ either targeted approaches in which for example specific antibodies are used for immuno-capture and/or immuno-detection of particular allergens on which the analysis is focused, or non-targeted approaches. Allergens in whole plants can be analysed based on their immunochemical and biological properties via Enzyme Linked ImmunoSorbent Assay (ELISA) and two dimensional electrophoresis in gel (2DE) followed by immunoblotting. Other methods, including novel mass spectrometry-based technologies are a potential alternative to the 2D-gel electrophoresis approach to multi-dimensional separation of proteins. In spite of their great performances, ELISA and 2DE in gels followed by immunoblotting do have some limitations and drawbacks with regard to the natural heterogeneity of some allergens (isoallergens), allergen extraction, matrix or processing effects, carbohydrates on allergens (CCD), analytical and detection method and availability of human sera. Analytical proteomics methods do also have some limitations with regard to the identification of proteins, the availability of antibodies against allergens and the identification of allergen-specific sequence tags.

Immunoassays such as ELISA and 2DE plus immunobloting require human sera from well clinically characterized allergic patients. These sera have to be available in sufficient quantities and sufficient number which might be difficult. The comparison of the allergen repertoire of GM and non-GM

organisms could include a pre-screening test with sera from experimentally sensitized animals as a substitute for allergic individual sera.

5.6.1. ELISA, 2D gel electrophoresis and immunoblotting

Conclusions

In spite of their great performances, ELISA and 2DE in gels followed by immunoblotting have some limitations and drawbacks which can be reviewed quickly by having a critical eye on each step of the method.

Limitations due to the sample

- Natural heterogeneity of some allergens: the major allergen Bet v1 appears usually in 2DE as more than 20 spots corresponding to isoforms. Most of the birch pollen sensitive individuals recognize all of them as allergens. However some allergic individuals do recognize only a subset of these molecules corresponding to a limited number of common epitopes scattered on the different isoforms of Bet v 1. A change in isoallergen content may be considered as an unintended effect of transgenesis.
- Allergen extraction from its crude extract and its solubility needed by the analytical 2 DE in gel: 2 DE in gel is performed in a solution of buffers in water. The analysed samples have to be soluble in these buffers. IEF is a very critical step of the method as it eliminates in the very beginning of the separation all small ions from the sample. If these ions are needed for the solubility of the sample this one will precipitate out of the liquid phase and will not migrate at its isoelectric point. The best improvement found to increase the solubility of the sample is to use a mixture of chaotropic agents (thiourea / urea) and detergents CHAPS. This solution may induce a modification of the isoelectric point of the sample as well as some alteration of its immunodetectability by antibodies originally induced *in vivo* by native samples. Another major limitation lies in the molecular sizes of the proteins that can be studied in the range of 10 to 200 kDa as a consequence of the use of poly acrylamide gel as support of the electromigration with a limited porosity.
Wheat flour contains watersoluble allergens as albumins and globulins. Poorly or non water soluble allergens are found in the gluten: gliadins and glutenins. They can be solubilized in thiourea - urea - CHAPS and thus be analysed in 1 and 2 DE gels.
- Matrix or process effect: allergenic samples coming from processed food may present added difficulties to be solubilized in water. Thermo labile epitopes may disappear during processing. Allergic individuals may be allergic only to raw shrimps, others only to boiled shrimps suggesting that the allergens were either the raw or the boiled shrimps respectively, involving in both cases different epitopes.
- Carbohydrates on allergens (CCD): many allergens are glycoproteins. Some allergic individuals do recognize glyco moieties by their IgEs. By immuno blotting in 1 or 2 DE in gel many glycolabelled molecules could be detected falsly as allergens. IgE antibodies are usually binding the glycated epitopes with low affinity without clinical relevance. A 2 DE gel followed by a blot incubated with these rather rare sera is able to tell that one is dealing with these CCDs: usualy only glycoproteins over 40kDa are detectable and the blot, instead of giving a few sharp allergenic spots, presents numerous fuzzy spots. Some glycoproteins like horse radish peroxydase which are heavily glycosylated may serve as positive controls to detect sera having IgE antibodies to CCD.

Limitations due to the analytical method

One of the major reasons to use purified allergens vs crude extracts in the ELISA and the allergen chip format is their binding onto the solid phase matrix. It is rather easy to bind a well known purified protein on a solid phase by using a given *ad hoc* chemistry. The amount of bound protein can be estimated. Conversely, a complex crude allergenic extract will be difficult to immobilize on a unique surface. A single chemistry will favour the binding of some proteins but be totally inefficient for other components which might be important allergens for a subgroup of allergic individuals. Chosing 2 major allergens efficiently immobilized by complementary chemistries may detect more allergic individuals than one single crude extract poorly immobilized.

2 DE in gel requires a skillful operator and has some essential manual steps, is rather time consuming and poorly reproducible. The use of precast gels and immobilised pH gradients contributes greatly to improve the reproducibility of the method.

Limitations due to the detection method

Quantification of allergens performed by enzyme immunoassays was a great progress, when it was introduced in 1972, over the previous method, the radio immuno assays. In the direct method, where allergens are immobilized and recognized by allergic individuals' IgEs, the binding of the enzyme labelled second antibodies to human IgEs may be highly dependent on the serum concentration used. Care should be taken to duplicate or triplicate these determinations by using several dilutions of serum. This effect of the serum dilution may be linked to the presence, in the serum, of inhibitory antibodies such as auto anti-IgE. They may create a steric hindrance phenomenon, an underestimation of the true IgE antibody concentration in the serum and impair the IgE binding to allergens. Finaly, an optimal choice of the length of time needed to make the optical density readings is important to try to keep a rather constant enzymatic activity upon time, an important contribution to the best IgE quantification.

Limitations due to the human sera available

In addition to ethical regulations that may limit the use of human sera, some technical limitations such as specificity of the sera used and reproducibility of new sera collected are to be mentioned.

Recommendations

1D and particularly 2D gel electrophoresis followed by immuno-blotting are appropriate *in vitro* tests to compare endogenous allergen expression in GMOs and their non-GM counterparts. However, if modifications are detected in the allergen repertoire and are considered to be of biological significance, more in depth sequence analysis is necessary to identify and quantify the allergens in question and their isoforms. In combination with immunodetection using allergic patient sera, mass spectrometry is an option to get sequence information and (semi)quantification specifically on allergen spots which differ between the GM and non-GM plant patterns.

For a comprehensive comparison of the allergenicity of the GM and non-GM plant using ELISA, 2D gel electrophoresis and the mass spectrometry based techniques, appropriate protein extraction procedures need to be tested to cover the different allergenic proteins present in the GMO and its counterpart, including those not easily soluble in aqueous buffers.

Immunoassays such as ELISA and 2DE plus immunobloting require human sera from well clinically characterized allergic patients. These sera have to be available in sufficient quantities and sufficient number which might be difficult. Therefore sera from experimentally sensitized animals that have been well characterized in terms of specificity and possible affinity could be used as probes for a prescreening of potentially allergenic proteins (see also Annex 6).

5.6.2. Analytical proteomics methods

Conclusions

2D gel electrophoresis followed by immunoblotting and MS analysis is a laborous method. A non-targeted 2D approach can be done by using DIGE (Difference in Gel Electrophoresis) in which all up-regulated protein spots are cut out of gel for identification. A drawback of 2D gelectrophoresis analysis is that highly charged and large proteins are difficult to resolve. Furthermore, low abundance proteins are also difficult to identify in the presence of highly abundant components. Extra fractionation of sequential extraction techniques is therefore needed. For MSMS identification of a protein spot from the 2D gel a relatively high amount of protein is needed.

Other methods may combine immunocapture when an appropriate set of antibodies is available and different kinds of MS analyses such as SELDI-TOF/MS, MALDI-TOF/MS or Q-TOF MSMS for identification of the bound fractions.

A new powerful *in vitro* analytical method is LC-QTOF/MSMS which has successfully been used to detect and identify major allergens in foods. This is a high throughput analytical technique which is sensitive, quantitative and not dependent on human sera. Since not many labs have the apparatus, expertise and skilled personel to perform this type of analysis yet, it appears as a promising technique in the future.
Other non-targeted techniques are available which allow to compare the protein expression patterns in extracts from GM plant and its non-GM counterpart (comparative LC-TOF/MSMS) and selectively perform MSMS on the differentiating mass peaks which might reveal unexpected variation in protein expression due to the genetic modification.
Isotope or mass tag labeling approaches and contemporary label-free spectral counting methods need to be assessed for suitability as non-targeted differential protein analysis tools for plant tissues.

Recommendations for further development

A comprehensive study of the specificity, sensitivity and feasibility of non-targeted profiling technologies based on "-omics" techniques, and particularly on proteomics for the assessment of allergenicity needs to be performed and a thorough assessment to be made of their advantages and weaknesses in order to compare these modern techniques with those of classical targeted analytical techniques in terms of reliability of interpretation of the results.

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ANNEX 6. ANIMAL MODELS

6.1. Introduction

Animal models of food allergy reproducing the pathophysiology of allergic diseases in humans provide a unique strategy for the *in vivo* detection and screening of proteins as well as chemicals with potential allergenicity. Allergic diseases in human encompass a wide variety of clinical symptoms that can manifest local reactions affecting the skin, the digestive and respiratory tracts as well as systemic reactions that can lead to an anaphylactic shock and even death. Although the pathophysiology of allergic diseases is still poorly understood, they can be distinguished by two major types of hypersensitivity reactions to one or more allergens, i.e. immediate-type hypersensitivity, (ITH) (associated with allergen-specific IgE antibodies in serum) or delayed-type hypersensitivity (DTH) (associated with allergen-specific effector T cells). The development of ITH and DTH reactions to a given allergen appears within minutes (ITH) to several days (DTH) after allergen exposure. In both cases, the symptoms reflect that the individual has been previously sensitized to this compound/allergen, although the exact time of sensitization cannot be precisely determined.

Animal models for food allergy have mainly been used for research purposes focussed on the basic mechanisms underlying food allergy, new strategies for prevention and therapy and for identification of reliable biomarkers and endpoints of the severity of an allergic reaction. They allow to better understanding the interactions between the numerous factors involved in the induction or suppression of oral tolerance, the relationship between digestibility and allergenicity, the influence of routes and doses of administration, duration and timing of exposure on allergic sensitization. Other factors contributing to the propensity to develop an allergic reaction such as the genetic background, the age of the subject, physiological, nutritional and environmental conditions at the time of exposure were also investigated. Animal models are also developed and evaluated as a systemic approach for hazard identification and allergy risk assessment; refined, standardized and validated test procedures are aimed to provide reliable tools for evaluating the relative potency of food proteins for sensitization and elicitation of allergic reactions (Bowman and Selgrade, 2009; Selgrade et al., 2003).
The different guidelines available for assessing the allergenicity of (novel) proteins actually focus on IgE mediated allergic reactions. They also particularly pertain to the capacity of a novel protein to trigger an allergic reaction in allergic consumers already sensitized rather than on the capacity to *de novo* sensitize predisposed atopic individuals.

Bioinformatics and IgE binding studies using sera of allergic individuals are key steps of the assessment of the possible sequence homology or structural similarity and cross reactivity of the newly expressed protein(s) in a GMO with known allergens. However, as due to several reasons the use of human sera is limited and the development of relevant and validated animal models for food allergy that can be used as a surrogate or complement is encouraged. They are also necessary because sensitization experiments can not be done in humans. In addition, *in vitro* tests cannot reproduce the complexity of the immune system and account for the interactions between the numerous factors that are involved in the regulation of the immune response and the development of a tolerance or allergic reaction to a given protein in particular conditions of environment and exposure.
Furthermore, other aspects may be taken into consideration when assessing the allergenicity of GMOs as previously emphasized in the document such as the immunogenicity and the adjuvanticity (see Annex 1.9.1).

Animal models should thus help in addressing 3 major issues:
i) is the novel protein a sensitizer, i.e. does it possess intrinsic properties that allow to *de novo* sensitize a predisposed individual?
ii) is the protein an elicitor i.e. is it able to elicit an allergic reaction in an already sensitized individual to the same or to a cross-reactive protein?

iii) is the protein an immunogen or an adjuvant, i.e. can it induce an immune response (non IgE) or facilitate/enhance the sensitization to another by-stander protein which is present together with the trait protein ?
In addition they also should allow a more holistic approach taking into account the impact of the food matrix, food processing and different conditions of exposure on the nature of the immune response which might be induced.
However, the relevance of using animal models for assessment of allergenicity of GMOs has been questioned because none of them completely reproduces the conditions of sensitization and clinical manifestations occurring in humans and because of their variability depending on the animals species and protocols used and finally for the lack of sensitivity and specificity (Goodman et al., 2008).
Combination, on a case by case basis, of *in vivo* testing using different animal models such as those described below with other tests may certainly improve the weight of evidence approach to evaluate the likelihood of allergenicity of a GMO.

6.2. Available animal models

6.2.1. Criteria for development of animal models for food allergy

6.2.1.1. Animal species and strains

Allergic individuals have a predisposed (*i.e.* atopic) genetic background and a close resemblance to this scenario in animal models is therefore desirable. This can be achieved by using a strain of an animal species that is susceptible for allergic disorders. For instance the Brown Norway rat is a high immunoglobulin - especially IgE - responder strain (Atkinson and Miller, 1994), the BALB/c mouse shows an immunological responsiveness, which has a propensity towards a Th2 type phenotype and IgE production (Hilton et al., 1997). Other strains of mice have also been extensively used including mutant strains [e.g. for expression or deficiency of specific toll like receptor (TLR)] or genetically modified animals. Alternatively, atopic dogs as well as neonatal swines are used as experimental models to test for specific IgE responses (Helm, 2002; Untersmayr and Jensen-Jarolim, 2006).
Besides genetic characteristics, other factors may be involved in the type and intensity of the immune response.The normal gut microbiota has been long recognized to play a major role in the differentiation of the intestinal immune system and particularly in the induction and maintenance of oral tolerance. It has been shown to affect the propensity of the mice to develop an allergic reaction when exposed to a foreign protein, which is increased in germ free animals as compared to conventional animals (Hazebrouck et al., 2009; Moreau and Gaboriau-Routhiau, 1996). Several mechanisms may be involved including the production of lipopolysaccharide (LPS). Indeed, feeding mice with ovalbumin (OVA) prior to i.p. immunization with OVA plus adjuvant, reduces OVA specific serum antibody production in normal C3H/HeN mice but not in C3H/HeJ which have a spontaneous mutation in the LPS responsive gene, subsequently identified as the TLR4 (see also below) (Moreau and Corthier, 1988).

6.2.1.2. Route and methods of administration

Several routes and modes of administration of the allergen are currently used. The oral route, using intragastric (i.g.) or oral gavage may be preferred over the intraperitoneal (i.p.), intranasal (i.n.) or cutaneous route, because it looks like a more relevant reflection of the situation in humans. Indeed, natural barriers such as the acidic and enzymatic conditions in the gastro-intestinal tract and the digestibility of proteins influence the allergenicity (Atkinson et al., 1996; Pauwels et al., 1979; Strobel and Ferguson, 1984; Turner et al., 1990; Untersmayr et al., 2003). However, exposure to proteins via the oral route normally leads to oral tolerance (Strobel and Ferguson, 1984; Turner et al., 1990). Also the possible dietary pre-exposure of the test animals or their parental generations to the protein under investigation may have resulted already in tolerance induction (Knippels et al., 1998). Oral sensitization to the protein may thus not always be achieved in this way and other routes of

administration are used to increase the sensitivity of the test. Proteins can be administered alone or in presence of adjuvants, such as cholera toxin (CT) for the oral route, complete or incomplete Freund's adjuvant (CFA/IFA) or alum for i.p. administration. Although natural adjuvant conditions such as intestinal inflammation or infections may occur in real-life situations, the use of adjuvants has been a point of discussion particularly when assessing the inherent allergenicity of a novel protein because it modifies the ability of proteins to induce sensitization. This may result in false positive results if appropriate controls are not included in the test and if the test is misused as the single definite way to conclude on the allergenic potential of a protein. However, with respects to the specific and limited objectives of the test the use of adjuvants may not be regarded as a problem in the context of the whole assessment procedure. Also, it is possible that trace amounts of LPS or various chemicals contaminating the allergens preparation exert some adjuvant effect and this could complicate interpretation of data generated using distinct allergen sources.
It is noteworthy that presence of the test protein in the animal chow would limit the sensitizing potential observed when an experimental sensitization is further performed.

In order to avoid false positive and false negative results due to the genetic background of the animal strain and/or conditions of exposure (e.g. diet, microbial environment, structure, presentation and dose of the allergen) appropriate controls should be included and the whole immune response (e.g. specific IgE and IgG antibody production and cellular response) explored.
The source and quality control required for compounds used as experimental allergens in animal models is a crucial question. This issue is also relevant when testing a purified newly expressed protein (as expressed in the plant or as a recombinant protein produced in microorganisms) and/or crude protein extracts from the whole crop for the allergenicity assessment of a GMO.

6.2.2. Models for investigating the sensitizing potential

The sensitizing potential of a protein is its capacity to induce the production of specific IgE antibodies. It is sometimes confused with its immunogenicity, i.e. the capacity to induce the production of any kind of antibodies and particularly those of the IgG class. Immunogenicity does not reflect an allergenic potential in the literal sense although it may be associated with a delayed type reaction and/or inflammatory diseases (Prescott et al., 2005). Models of experimental sensitization are mainly developed in mice and rats, using different procedures. They have been used for the assessment of allergenicity of purified proteins, of protein extracts from foods and also of complex whole foods. Other non-rodent animal allergy models like pigs and dogs are often introduced as they show a more related physiology and immune system similarity to humans (Helm and Burks, 2002) and may therefore contribute further to the knowledge of the allergic responses in humans (Kimber et al., 2003). For review see also Dearman and Kimber (2009).
Different strains of mice such as BALB/c or C3H mice and Brown Norway (BN) rats are particularly used because of their natural propensity to develop a Th2 type immune response which could resemble a human atopic genetic background.

6.2.2.1. Mice

Dearman and Kimber developed a model in which 8 to 12 week old adult female BALB/c mice were used. Sensitization was achieved by repeated i.p. administrations of a test protein dose in phosphate buffered saline (PBS), without adjuvant. The dose depends on the expected allergenicity of the protein. On day 14, 28 or 42 mice blood samples are tested for protein specific antibody determinations (Dearman and Kimber, 2001; Kimber et al., 2003).

The degree of sensitization is estimated by the quantification of the IgE (and IgG1) antibodies that have been produced after the experimental sensitization with the test-protein, the capacity to induce a specific IgE response in a large proportion of treated animals seems to reflect the sensitizing potential in humans. Using this model experiments have been performed with OVA and bovine serum albumin (Dearman et al., 2000), lipase from the mould Aspergillus oryzae (Hilton et al., 1997), the minor

peanut allergen: peanut agglutinin and the non-allergenic potato acid phosphatase (Atherton et al., 2002; Dearman and Kimber, 2001). These experiments show difference in the vigor of specific IgE antibody responses induced by OVA and bovine serum albumin (Dearman et al., 2000). Serological responses appear to distinguish between strong-, weak- and non-allergenic proteins, with a pattern of IgE responses that is consistent with what is known of the relative sensitizing potential of the corresponding proteins in humans (Atherton et al., 2002; Kimber et al., 2003).

Adel-Patient et al. (2000; 2001) quantified the intensity and analysed the specificity of IgE, IgG1 and IgG2A antibody responses in BALB/c mice that were i.p. immunized against β-lactoglobulin (BLG), a cow's milk allergen. They showed that the IgE epitopes on BLG recognized by the mouse were the same as those recognized by the serum IgE antibodies of milk allergic humans.

In other models, developed for food allergy, the mice showed apart from antibody responses (IgE, IgG1) also clinical symptoms of food allergy such as diarrhoea, anaphylaxis, and eosinophil and mast cell accumulation (Prescott and Hogan, 2006).

Gizzarelli et al. (2006) sensitized BALB/c mice by i.g. gavage of protein extracts from a wild type and an herbicide tolerant genetically modified (GM) soybean in the presence of cholera toxin. They indeed induced Th2 immune responses that were similar for both kind of soybeans but did not evidence any specific response to the newly expressed protein in the GM soybean.

The intranasal route has also proven to be an efficient route for sensitization in BALB/c mice (Hilton et al., 1997). In addition, BALB/c mice have also been used to assess the allergenicity of whole food (e.g. milk, peanut) and not only of purified proteins. Adel-Patient et al. (2005) showed that the allergens recognized by mouse IgE antibodies were the same as those recognized by serum IgE antibodies of humans allergic to milk or peanut.

The model developed by Strid et al. (2004) is derived from those described above, whilst some essential parts have been modified in the procedure. Sensitization is achieved on 6 to 8 week old female BALB/c mice using first a single i.g. administration of the protein antigen in PBS followed by an injection of the protein in presence of complete Freund's adjuvant (CFA) and, on day 28, a boost injection of the protein solution in PBS is performed in the foot pad. Oral challenge of sensitized mice is performed using high doses of protein, in the hundreds milligrams range. This test has been used to assess the delayed type hypersensitivity by measuring the food pad swelling after the last boost administration. Cell proliferation and cytokine production in spleen and lymph node cells are measured by ELISA, after in vitro re-stimulation. Total and specific IgE and specific IgG, IgG1, IgG2a were measured using immuno-assays. Performed with both peanut and egg allergens, this model confirmed that the the estimated sensitizing potential did depend upon the nature of the protein.

In a subsequent paper by Strid et al. (2005) it was concluded that induction of oral tolerance to peanut protein can be prevented by epicutaneous exposure to peanut protein, which even might modify existing tolerance to peanut. The epidermal exposure to protein allergens would selectively drive Th2-type responses and might promote sensitization to food proteins upon oral (gastrointestinal) exposure. Similar findings were made by Adel Patient et al. (2007) thus confirming the clinical observations on humans allergic to peanut and the fact that exposure via different routes may interact and greatly affect the sensitization.

The oral sensitization to OVA was studied in different mice strains (e.g. BALB/c, B10.A and ASK) and rats by Akiyama et al. (2001). In the different mice strains the effect of age, oral feeding technique and dose of protein were examined. In terms of OVA-specific IgE and IgG1 antibody, the B10.A mice were found to exhibit the highest response of the three mice strains tested. Based on the results observed it was also suggested that oral sensitization of mice requires low doses and intermittent protein intake. Bodinier et al. (2009) have tested the sensitizing potential of wheat gliadins in 3 strains of mice and concluded that BALB/c mice were the most appropriate model.

Bowman and Selgrade (2008) subcutaneously (s.c.) or orally sensitized C3H/HeJ mice with extracts from most common allergenic foods (e.g. peanut, Brazil nut and egg white), and from non-allergenic foods such as turkey and spinach in the presence of cholera toxin. The aim was to establish a spectrum of potency of the tested food allergens in order to relate the allergenic potency of a novel protein to known food allergens. The oral route of exposure appeared to better discriminate allergenic foods from non-allergenic than the s.c. administration.

Birmingham et al. (2007) proposed an adjuvant free transdermal sensitization protocol on mice with subsequent oral challenge and measure of the antibody response and clinical score for anaphylaxis. Extracts from most common allergenic food (e.g. peanut, tree nut, egg, fish, milk etc ...) and from non-allergenic food (e.g. spinach, pinto bean, sorghum etc...) were used which allowed to test the sensitivity and specificity (e.g. positive and negative predictive values) of this model.

Transgenic animal (mouse) models

The use of GM animals, particularly mice, for the assessment of allergenicity is based on the rationale that food allergy is the consequence of altered oral tolerance i.e. a physiological mechanism of immune suppression to environmental allergens. Indeed, the mucosal-associated immune system (MALT), and in particular the intestinal immune system is geared toward induction of immune tolerance to exogenous but harmless antigens, such as dietary antigens and components of the bacterial flora to which epithelial tissues are constantly exposed (Dubois et al., 2003). This process prevents the induction of T cell–mediated inflammatory and allergic reactions that primarily affect surface epithelial tissues (buccal mucosa, intestine, skin). Immune tolerance induced by the oral route, prevents the outcome of both systemic or local allergic reactions to proteins and chemicals and is induced and maintained by regulatory T cells (i.e. Treg) (Desvignes et al., 1996; Desvignes et al., 1998; Desvignes et al., 2000; Dubois et al., 2003). Most animal models and particularly transgenic mice aim at circumventing the mechanisms of normal oral tolerance.

The major histocompatibility complex (MHC) plays an important role in the development and polarization of the immune response which may result in strain dependent qualitative and quantitative antibody responses to experimental sensitizations.
Several strains of mice such as the different C3H variants have been used to study the influence of the genetic background in the assessment of the sensitizing potential of a protein. Altered IgE responses to OVA were thus observed in C3H/HeJ, TLR4 deficient and thus LPS-hyporesponsive, strains of mice (Vaz et al., 1971). Using 3 different substrains of C3H mice with the same genetic background (H-2k), Kaiserlian et al. (2005) have demonstrated that repeated oral immunizations with BLG in presence of CT induced variable clinical scores of immediate-type hypersensitivity upon oral challenge with BLG alone, reproducing the variable allergic disease severity in human. Wild type normal C3H/HeN mice did not develop clinical symptoms, C3H/HeOuJ mice (a substrain of HeN mice) could be sensitized after several immunizations but developed only mild clinical symptoms, while C3H/HeJ mice could be rapidly sensitized and developed severe systemic symptoms with anaphylaxis. Remarkably, depletion of natural Treg increased the severity of symptoms in C3H/HeOuJ mice, which became as susceptible as C3H/HeJ mice.

Other studies have pointed out that the C3H/HeJ mouse strain is more susceptible to the induction of food allergy than other non deficient mouse strains which makes it a valuable strain for food allergy research (Frossard et al., 2004a; Frossard et al., 2004b; Kaiserlian et al., 2005; Sicherer and Sampson, 2006). However studies of Bashir et al. (2004) and Berin et al. (2006) on several mouse strains of different genetic backgrounds including the $TLR4^{+}$- and $TLR4^{-}$-C3H, C57Bl/6 or BALB/c mice sensitized either with BLG or peanut extract demonstrated that T-cell responses, propensity to develop an allergic reaction and intensity of the IgE response were under H2-linked genetic control and depend on both the TLR4 status of the mice and the nature of the antigen.

Advantages of application of transgenic animals to hypersensitivity studies may be that different types of genes, the gene product of which influences the immune system, might be introduced or knocked out of the genome, and the consequences of the genetic alterations studied. In addition of genetic variants of some mouse strains such as the C3H mice, genetically modified mice with impaired immune tolerance may also be a useful tool for the assessment of allergenicity.

Data from selected gene-modified mouse have confirmed that Treg play an important role in controlling intestinal homeostasis. Indeed, mice with a gene defect affecting Treg number or function develop spontaneous chronic inflammatory bowel disease and enteropathy. The Treg compartment comprises natural $CD4^+CD25^+Foxp3^+$ Treg, (which constitutively account for 5-10% of peripheral $CD4^+$ T cells) and antigen inducible or antigen-experienced $CD4^+CD25^-Foxp3^-$ Treg. Both types of Treg can perform immune suppression of both antigen-specific T and B cell responses, by controlling the priming of naive T or B cells in secondary lymphoid organs (spleen, lymph nodes) as well as the activation of antigen-specific/memory effectors in target organs or epithelial tissues. Goubier et al. (2008) showed that subsets of dendritic cells in mice are tolerogenic and that depletion of these cells with specific monoclonal antibodies impairs oral tolerance and renders mice susceptible to *in vivo* sensitization by the oral route, due to lack of suppressive mechanisms.

Some mouse models with targeted disruption of a single gene essential for Treg differentiation, activation or function and which exhibit gut inflammation due to breakdown of oral tolerance to the flora and/or dietary antigens are also available. These mouse models include: i) MHC class II (Cosgrove et al., 1991) and invariant chain (Ii) (Viville et al., 1993) mice, which respectively harbour a complete or partial defect in class II-restricted $CD4^+$ T cells (due to impaired positive selection in the thymus); ii) IL-2α chain (CD25) and IL-2R (Desvignes et al., 1998) KO mice, in which survival and expansion of Treg is impaired; iii) IL-10 (Desvignes et al., 1996) and TGFß (Cosgrove et al., 1991) KO mice, in which Treg function is altered; iv) TcRaß KO mice, which lack T cells and NK-T cells; v) Foxp3 KO mice, which have no natural Treg (Hori et al., 2003)

In the future, two types of transgenic animal models are likely to become of most interest in the search for GMO allergenicity. The first one uses humanized mice in which major histocompatibility class II complexes are entirely of human origin. These will help understanding how a GMO is processed for presentation to T cells. Mice carrying specific human T cell receptors are also available, which will be useful to evaluate the signalling provided to T cells by GMO presentation. The second model is based on the rationale that allergens interact with the innate immune system via multiple mechanisms, which constitute the first event occurring when an allergen comes into contact with a living body. The innate immune system is highly conserved on an evolutionary basis. The use of some of the many mouse strains made deficient in innate immunity components (for example Toll or NOD receptors) will provide information likely to be directly extrapolated to the human situation.

Finally, promoting new developments in constructing transgenic animals for allergenicity testing is in accordance with the "three R concept" (Replacement, Reduction, Refinement) based on finding replacements for conventional laboratory mice, reduction of amount of laboratory animals and use of modern technology.

6.2.2.2. Brown Norway rat

Another important animal model of food allergy is the Brown Norway (BN) rat (Jia et al., 2005; Pilegaard and Madsen, 2004).

The BN-rat is a high immunoglobulin - especially IgE - responder strain, which resembles high IgE responsiveness of atopic individuals.

As reported by Atkinson and Miller (1994) and Miller et al. (1999), 6 to 8 week old male BN rats were sensitized by i.p. injection of 10 µg of protein together with carrageenan used as adjuvant. They demonstrated that the pattern of cow's milk allergens recognized by sensitized rat IgE antibodies was the same as the allergen repertoire in allergic humans to milk. Moreover the IgE specificity at the

epitope level was also very similar. This model was developed as a sensitive model for the investigation of allergic reactions to food and to determine the impact of dietary factors on the development of oral sensitization. It was not particularly aimed for predicting the allergenicity of new proteins.

This model has also been used to study oral sensitization to allergenic foods or purified food proteins upon administration by gavage of 4 to 6 week old male BN rats in the absence of adjuvant (Knippels and Penninks, 2005; Knippels et al., 1999a; Knippels et al., 1998; Knippels et al., 1999b; Knippels et al., 2000). In a comparative study with Wistar, Hooded Lister, PVG and BN rats, the latter was found to be the most suitable strain of rats for oral sensitization (Knippels et al., 1999b). The outputs of the study by Pilgaard and Madsen (2004) have shown that female rats are more appropriate than male for sensitization studies.

The first experiments in this model, developed in particular for the prediction of potential allergenicity of proteins, were performed using OVA (Knippels et al., 1998). In subsequent studies, a whole food (cow's milk), whole protein extracts of hen's egg white (Knippels and Penninks, 2002; Knippels et al., 2000) and peanut, and purified strong-allergenic (peanut Arah1 and shrimp Pen a1), weak-allergenic (potato Sol t1) and non-allergenic (beef tropomyosin) proteins have been used (Knippels and Penninks, 2003). Upon oral application by gavage, rats showed specific IgE and IgG-antibodies to OVA (Knippels et al., 1999b), hen's egg white proteins and cow milk proteins (Knippels et al., 2000). Temporary decrease in breathing frequency, blood pressure, and increase in gut permeability, which resembles human clinical manifestation, was then observed after oral challenge with OVA (Knippels et al., 1999a). Exposure to complex protein mixtures (cow milk, hen egg white) also showed IgE antibody responses to a comparable selection of proteins as observed in allergic patients (Knippels et al., 2000).

The results obtained with the BN rat indicate that it might be a useful animal model to assess the potential allergenicity of novel food proteins. However, a high variability is observed in the induction of an IgE response after a well defined protocol of sensitization. This is due to inherent variability of the strain, to environmental conditions to the diet of the animals and their parental generations and to the nature of the sensitizing antigen. Despite the use of appropriate positive and negative controls, this may hamper to conclusively predict the allergenic sensitizing potential of a novel protein.

6.2.2.3. Guinea pig

The guinea pig model is by far the oldest animal model for allergenicity. It has been used for experiments concerning the allergenicity of chemicals, cosmetics, pharmaceuticals and in particular for oral sensitization studies of cow's milk and infant formula (Devey et al., 1976; Fritsche, 2003; Kitagawa et al., 1995; Piacentini et al., 1994). However it is a less frequently used model for the assessment of allergenicity of novel proteins. The guinea pig can be sensitized by the oral route without adjuvant but immunologic reactions to proteins are different of those occurring in humans; for instance reaginic antibody responses are of the IgG1a subtype (Fritsche, 2003). Drawbacks for the further use of the guinea pig in food allergy research are therefore the significant differences in immuno-physiology when compared with other species, the limited knowledge of its immune system and as a consequence the lack of tools to study its immune system, and finally its questionable specificity in allergic sensitization tests.

6.2.2.4. Non-rodent animals

In several non rodent species, other animal models for food allergy have been developed. As examples, the antibody responses observed in dogs and pigs experimentally sensitized to milk or wheat flour (Buchanan and Frick, 2002) or to peanut and different nuts (Helm et al., 2002; Teuber et al., 2002) partly mimic the pattern of recognition of human IgE antibodies and the elicited clinical manifestations partly reproduce those occurring in allergic humans.

In the following paragraphs these models in dogs and pigs are presented.

6.2.2.4.1. Atopic Spaniel/basenji dogs

The dogs that have been first introduced to test for food allergy are derived from a colony of inbred spaniel/basenji dogs, which have a genetic predisposition to allergy and have history of sensitivity to pollen and foods (Buchanan et al., 1997; Buchanan and Frick, 2002; Ermel et al., 1997; Jeffers et al., 1996). This resembles high IgE responsiveness of allergic patients. Next to this the dog is one of the few species other than humans in which food allergies develop naturally and show the same clinical symptoms (Buchanan and Frick, 2002; Paterson, 1995). A similar model further developed by Buchanan and Frick (2002) uses newborn spaniel/basenji type pups. Sensitization starts one day after birth by s.c. injection of the protein extract with alum as adjuvant and a live vaccine. Then pups are boosted by s.c. injections of 10 µg of protein in alum at bimonthly intervals. Allergic responses are tested by skin tests and feed challenge. The allergic response is determined by measuring the size of the wheal. The allergic response measured by feed challenge is performed by scoring of vomiting and number and quality of stools after oral challenge of the dogs with the protein (Buchanan and Frick, 2002; Teuber et al., 2002). Sera from the animals 1, 2 and sometimes 3 years old were used for IgE immunoblotting (Teuber et al., 2002).
This model has been performed using proteins from wheat, cow's milk and beef extract (Buchanan and Frick, 2002) and also peanut, walnut, Brazil nut, barley and soy (Teuber et al., 2002). In the last experiment the hierarchy of reactivity by skin testing is similar to the clinical experience in human subjects (i.e. peanut, tree nut, wheat, soy, barley). Sligth cross-reactivity between walnut and Brazil nut was the only case of cross-reactivity (Teuber et al., 2002).

6.2.2.4.2. Neonatal swine

The model develop by Helm et al. (2002) uses newborn piglets from Large White/Landrace sows. In the optimal sensitization protocol the piglets were i.p. sensitized on days 9, 10 and 11 after birth and boosted the same way on day 18 and 25. Sensitization was performed using crude peanut protein extract in presence of cholera toxin.
After i.g. challenge, the allergic response was measured using an evaluation of clinical manifestations (e.g. cutaneous, respiratory and digestive symptoms), skin testing after intradermal injection of protein and histologic examination of the digestive tract. In serum, specific IgG antibodies were detected by ELISA whereas specific reaginic antibodies were determined by PCA (Helm et al., 2002).
The physiological and immunological characteristics of pigs are similar to that of humans (Murtaugh, 1994). They closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity. However, piglets are described to be immunodeficient at birth and highly dependent upon colostral immune factors delivered from the sow (Stokes et al., 2004). Such passive immunity of newborn piglets is followed by a complete development of the immunity. The final maturation of the intestinal epithelium reaches the stage of an adult pig after seven weeks (Machado-Neto et al., 1987; Stokes et al., 2004). Therefore, neonatal piglets have an anatomy, nutritional requirements and other characteristics of the digestive tract that are similar to those of the newborn human infant (Helm et al., 2003; Untersmayr and Jensen-Jarolim, 2006). Main physical symptoms after oral challenge are similar to those observed in humans. Moreover histology of the digestive tract shows architectural abnormalities similar to those observed in intestinal mucosa of allergic individuals (Helm et al., 2002). Due to limited experimental availabilities the presence of reaginic antibodies could only be suggested after exposure to peanut proteins. This model has been tested with peanut proteins (Helm et al., 2002) and recently with the chicken ovomucoid (Rupa et al., 2008).

6.2.2.5. Conclusion

The models described above mainly concern the sensitization of the animal to a certain protein, which is a most important part of allergenicity testing. When a protein is able to provoke sensitization, it most likely can cause allergic reactions upon re-exposure. This is the reason why most models mainly focus on the sensitizing potential of proteins. Still, although considerable progress has been made it is clear that none of the developed animal models is currently sufficiently evaluated, validated and widely accepted

6.2.3. Animal models for investigating the elicitation potential

The assessment of the potency of an allergenic protein/food to elicit an allergic reaction is normally studied in animals that have previously been experimentally sensitized to the protein or the whole food, using either an i.g. or an i.p. route in presence or absence of adjuvant, in order to qualitatively and quantitatively optimize the IgE response. Challenges are then realized in order to reproduce the characteristics of the allergic reaction in humans at a serological and cellular level and also with regards to systemic clinical manifestations. The challenge may be performed using the sensitizing protein either under its native structure or after modification, e.g. by processing, a cross reactive protein or the whole food in which the protein is present. The main animal models are mice and rats but guinea pig and, more recently baby pigs are also used.

The model developed by Li et al. (1999; 2000) used 3 to 5 week old female C3H/HeJ mice. The mice were sensitized on day 0 and boosted five times at weekly intervals, by intragastric gavage of protein in presence of cholera toxin as adjuvant. On day 42 the mice were i.g. challenged with two doses of protein and specific IgE antibodies were measured in blood samples. After challenge clinical symptoms occurred including increased vascular permeability, hyper-permeability of the gut mucosa, lung inflammation and systemic anaphylaxis symptoms such as anaphylactic shock. They were evaluated with a standardized scoring system ranging from no symptoms to death. Other allergic responses were determined by for example detection of vascular leakage, determination of plasma histamine levels and cutaneous mast cell degranulation. The model mimics the clinical and immunological characteristics of peanut and cow's milk allergy which involve multiple organs in human allergic individuals.

Such a model thus allows to analyze different mechanisms involved in the immune-pathology of an IgE-mediated allergic reaction, to measure and to grade the severity of the reaction according to the serological and clinical manifestations it induces and to study the impact of any change in the structure of the protein on its allergenicity. This is an important matter since the production of a heterologous foreign protein in a GMO might result in structural modifications.

Adel-Patient et al. (2003) developed a BALB/c mouse model which reproduced the manifestations observed during the allergic reaction to BLG and showed that, after challenge, the biochemical and clinical manifestations occurring during both the early and late phase of the allergic reaction differ and are activated through different metabolic pathways depending on the structure (i.e. native *vs.* denatured) of the allergen.

Other studies in mice (BALB/c, C3H/HeJ) and rat (BN) models have dealt with more mechanistic, therapeutic and prophylactic aspects of allergenicity and have been summarized by Knippels et al (2004).

6.2.4. Models for investigating the adjuvanticity

In vivo testing of adjuvanticity in relation to food allergens have been successfully performed in a number of laboratories using oral immunization protocols with cholera toxin as adjuvant (Li et al., 2000), often with slight modifications of the original procedure to ensure good responses (e.g. Vinje et al., 2009). The cholera toxin model can serve as a fairly reliable positive control. Peanut extract or purified allergenic proteins can be used as a standard allergen. Specific IgE in serum are determined by ELISA or PCA – the former test determines specific IgE antibody levels, while the latter test indicates biologically functional IgE levels (for references, see Vinje et al., 2009). Clinical anaphylactic reaction can also be used as an outcome (Li et al., 2000; Vinje et al., 2009). To be able to detect a possible weaker adjuvant effect than that of cholera toxin, one may want to use more extensive immunization schedules (Brunner et al., 2009; Scholl et al., 2005; Untersmayr et al., 2005; Untersmayr et al., 2003). They showed that anti-acid treatment enhanced the IgE response to oral

administration of fish and hazelnut allergen in mice and also evidenced the adjuvant effect of aluminium hydroxyde. A similar effect was also observed in humans (Scholl et al., 2005; Untersmayr et al., 2005).

Animal models may also be used to assess the adjuvanticity of a newly expressed protein in a GMO by comparing the Th1 and Th2 immune responses induced by a known allergen (e.g. OVA) in presence or absence of the protein in question. The adjuvanticity of Cry proteins (i.e. insecticidal proteins from *Bacillus thuringiensis*) has thus been demonstrated in mice (Moreno-Fierros et al., 2003; Vazquez-Padron et al., 1999). Prescott et al. (2005) reported that a recombinant form of the bean α amylase inhibitor was expressed in a GM pea with post-translational modifications (i.e. in the glycosylation pattern) which may result in an increased antigenicity and adjuvanticity. Recently using the BALB/c mouse model, Guimaraes et al. (2008) have shown that the adjuvanticity of the Cry 1Ab protein as expressed in the genetically modified MON 810 maize was much lower than previously described and that the mechanism of action would anyway be different from that of the cholera toxin.

6.3. Conclusions and recommendations

Most animal models are designed in such a way that they circumvent, or at least, try to circumvent the mechanism of oral tolerance.
Some have an increased susceptibility for allergic disorders, i.e. the BN-rat model, the BALB/c mouse model, the neonatal swine model and the spaniel/basenji type dog model. They may also reproduce, at least partially, the initial changes occurring at the intestinal epithelium and the symptoms that accompany the allergic reaction.
In addition, several transgenic mouse models have been developed and are available for the study of the factors and mechanisms of (de)regulation of the immune response and induction of allergy.
A huge variability is observed in allergic responses of humans which may vary depending on individual susceptibility based on genotype, exposure and environmental conditions. Like in allergic humans, the different animal models reflect the influence of the genetic background on the susceptibility for the development of the allergy. However, no "ideal" animal model can mimic the heterogeneity of the population of allergic people nor take into account all those factors that interact in the induction or repression of an allergic reaction to a protein and consequently reproduce the conditions of development of an allergic reaction to food in human consumers. Several animal models, including transgenic mice, can contribute to a better understanding of the different mechanisms underlying the sensitization and elicitation which may provide useful additional tools in the risk assessment process but a single model is probably not sufficient to cover all requirements for the prediction of allergenicity of novel proteins and GM foods. An option would be a combination of models with different characteristics and procedures, selected on a case-by-case basis and integrated in the whole assessment process to provide a reliable evaluation of the frequence and severity of allergic reactions to a novel protein with acceptable sensitivity and specificity.
In addition, combining transgenic mouse models with other approaches for allergenicity testing could be a powerful strategy in the future in order to maximise and better characterize the immune response in particular conditions. In conjunction with -omics technologies studies on transgenic mice could establish a very sensitive assay system using dosages characteristic of human exposure to identify potential allergens, and lead to less use of human material and experimental animals.
Finally, to date no single animal model is available for assessing the allergenicity and particularly the potential to *de novo* sensitize atopic individuals of a novel protein or a novel food derived from a GMO. Animal models are not conclusive per se but they can provide useful information on the different mechanisms underlying the induction and development of an allergic reaction.

<u>Conclusions</u>
Animal models may be used for assessing the potential of a novel protein to elicit an allergic reaction in individuals already sensitized when there are indications of a possible cross reactivity with a known allergen based on the origin of the source and/or the outcomes of bioinformatics studies. Different models of animals that can be efficiently experimentally sensitized before being challenged with the

test protein are available. All the different animal models available (e.g. different species and/or different procedures of sensitization) have advantages and pitfalls that preclude any single test to provide definite conclusions. However, they may be used in combination in order to reproduce as much as possible the different situations of exposure and reaction in at risk groups of the population and thus improve both the sensitivity and specificity of the assessment.
Animal models could be used in addition to or as substitutes of IgE binding studies using allergic human sera for assessing the allergenicity of the newly expressed protein or the whole GM plant although sera from clinically well characterized allergic individuals are the reference material. In the case when the host of the genetic modification is a known food allergen, sera from animals experimentally sensitized in appropriate conditions could provide useful information when used in western blotting studies for qualitative and quantitative analysis of the allergen repertoire (i.e. the pattern of endogenous allergens) of the GM plant *vs.* the non GM comparators.

In case there is indication from the origin or from the structure of the newly expressed protein that it might act as or like a sensitizer, the potential of the newly expressed protein to sensitize *de novo* atopic individuals could be experimentally investigated only on animal models. Various animal models (e.g. using different animal species, including transgenic mice, and different procedures of immunization) are available to study the polarization of the immune response toward a Th2 type response and the development of an allergy. In many cases, animals respond to the same epitopes and in the same way as it is found in humans. However the value of any animal model in the prediction of allergenicity of a food protein in humans has not been validated. The variability of the human individual immune response and conditions of exposure that may be encountered preclude that a single test could be used to predict with a sufficient sensitivity and specificity whether or not any protein is likely to be a sensitizer in the real life conditions of exposure.
As discussed above useful information could however be obtained by combining different models in order to reproduce as much as possible the genetic background as well as different environmental factors and conditions of exposure of at risk groups of the population on a case by case basis. The sensitization after experimental immunisation should be assessed by a comprehensive analysis of the immune response in animals and confirmed upon challenge with the test protein.
The same general considerations also apply for the assessment of immunogenicity and adjuvanticity of food proteins.
Toxicological studies using multi transgenic mice have brought significant progress in understanding the physiological mechanisms of the response to xenobiotics (Hwang et al., 2001). Therefore it is also expected that the use of multitransgenic animals in allergenicity testing will help in better understanding the role of different factors in the process that results in allergic reaction.

Recommendations
Antibodies obtained from animals experimentally sensitized in appropriate and well defined, conditions could be used as a substitute for allergic human sera for a (pre)screening of the immunological cross-reactivity of the newly expressed protein with known allergens.
They could also provide useful information when used in western blotting studies for qualitative and quantitative analysis of the allergen repertoire of the GM plant as compared to the non-GM counterpart.
In specific cases such as when indication for sensitization or adjuvant potential exists, additional information gained from (combination of) animal models might be useful for clarifying the mechanisms involved and the possible consequences in terms of safety of the newly expressed proteins.

Recommendations for further development:
Future work should aim at improving the sensitivity and specificity of tests on animal models as to allow consistent and reliable conclusion on a sensitizing and/or adjuvant potential and explore the use of transgenic animals which are likely to develop *de novo* sensitisations to newly expressed proteins and are extrapolable to human situation.
Different species and/or different procedures of immunization/sensitization may be used in combination, including in the presence or absence of an adjuvant, in order to reproduce as much as

possible the different situations of exposure and reaction observed for at risk groups of human consumers and thus improve the sensitivity and specificity of the test. A complete analysis of the whole immune reaction occurring after challenge should then be performed including observation and measurement of clinical and biological symptoms. In addition, challenges of experimentally sensitized animals with different doses of the newly expressed proteins could be performed using either purified proteins or whole protein extracts from the GMO in order to assess the impact of possible interactions between the newly expressed protein and the food matrix on the potential of elicitation.
When there is no information on the novel protein or on the food derived from a GMO or when specific situations are to be assessed such as the allergy risk in young children (see Annex 1.8), specific tests might be developed. In such cases, the use of animal pups or of transgenic animals in which immune barriers have been abolished to facilitate the development of an immune response towards an allergic sensitization could be considered in the risk assessment process.

It is unlikely that any single animal or GM animal model, will be appropriate for all testing situations. Nevertheless, transgenic mouse models possess characteristics reflective of current understanding of particular aspects of allergenicity and could give useful even if limited information. In a prospective way it is recommended to further develop transgenic animal models for allergenicity testing not only with single genes inserted or deleted from the animal genome but also containing multiple gene cassettes to modify the ability to develop allergenicity more profoundly.

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ABBREVIATIONS

- Ab: antibody
- Ag: antigen
- APC: antigen-presenting cell
- BLAST: Basic Local Alignment Search Tool
- CBB: Coomassie Brilliant Blue, staining
- CCD: Cross-reacting Carbohydrate Determinant
- CD63; CD203c:
- CE: capillary electrophoresis
- CF: chromatofocusing
- CV: cross validation
- CZE: capillary zone electrophoresis
- DBPCFC: Double Blind Placebo Controlled Food Challenge
- 2DE: two dimensional electrophoresis
- DIGE: Difference in Gel Electrophoresis
- DTH: delayed-type hypersensitivity
- EAST: Enzyme Allergosorbent Test
- ELIFA: Enzyme Linked Immuno Filtration Assay
- ELISA: Enzyme Linked Immunosorbent Assay
- EST: expressed sequence tags
- GI: gastrointestinal
- IEF: isoelectric focusing
- IFN: Interferon
- Ig: Immunoglobulin
- IL: Interleukin (IL-1, IL-2…)
- ITH: immediate-type hypersensitivity
- LC: liquid chromatography
- LC-ESI-IT-MS/MS: liquid chromatography-electrospray ion-trap tandem mass spectrometry
- LC-ESI-MS: Liquid Chromatography Electron Spray Ionisation Mass Spectrometry
- LC-MS/MS: Liquid Chromatography Mass Spectrometry
- LC-MS: liquid chromatography-mass spectrometry
- LTP: lipid-transfer-protein
- MALDI-MS: Matrix Associated Laser Desorption Ionisation Mass Spectrometry
- MEME: Multiple EM for Motif Elicitation algorithm
- MHC: Major Histocompatibility Complex
- MS: mass spectrometry
- NOD: Nucleotide oligomerisation domain
- nsLTP: non-specific lipid transfer proteins
- PAG: poly acrylamide gel
- PAGE: polyacrylamide gel electrophoresis
- PBMC: Peripheral blood mononucleated cells
- pI: isoelectric point
- PMF: peptide mass finger printing
- Q-TOF-MS/MS: Cut of Time Of Flight Mass Spectrometry
- RAST: Radio Allergosorbent Test
- RBL: Rat basophil leukemias
- ROC: receiver operating characteristic (ROC) curves
- RP: reversed phase
- SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis
- SELDI-MS: Surface Enhanced Laser Desorption Mass Spectrometry
- SELDI-TOF MS: surface-enhanced laser desorption/ionization time of flight mass spectrometry

- SGE: slab-gel electrophoresis
- SGF: simulated gastric fluid
- SPT: skin prick test
- TGF: Transforming Growth Factor
- Th cells: T-helper cells
- TLR: Toll-like receptor

GLOSSARY

- Adjuvants: substances that, when co-administered with an antigen, increase the immune response to that antigen.
- Allergen repertoire: the pattern of distributions of endogenous allergens.
- Angio-edema: the swelling of the dermis, subcutaneous tissue, mucosa and submucosal tissues.
- Antigen presentation: the process by which certain cells in the body (antigen-presenting cells) express antigen on their surface in a form recognizable by lymphocytes.
- B cell: lymphocytes that develop in the bone marrow in adults and produce antibody.
- Basic Local Alignment Search Tool (BLAST): a computer program for comparing DNA and protein sequences.
- Basophils: granulocytic white blood cell with cytosolic granules that stain with basic dyes and contain biologically active mediators responsible for the clinical manifestations of the allergic reaction.
- CAP system: a solid-phase quantitative immunoassay for measuring allergen-specific IgE in human serum.
- CD markers: surface molecules of leukocytes cells used to differentiate cell populations.
- Coeliac disease: an autoimmune disorder of the small intestine that occurs in genetically predisposed people.
- CpG motifs: components of bacterial DNA.
- Cross-reactivity: when sensitisation to one allergen causes the immune system to respond to another allergen because of shared epitopes (identical or with a high degree of similarity) between the allergens.
- CTLA-4: a protein that plays an important role in the regulation of the immune system. It is also known as CD152.
- Cytokines: a generic term for soluble molecules that mediate interactions between cells.
- Dendritic cell: a set of cells present in tissues that capture antigens and migrate to the lymph nodes and spleen, where they are particularly active in presenting the processed antigen to T cells.
- E()-value: an alignment derived from a FASTA search of a database is accompanied with an E()-value, which represent the number of times the corresponding alignment score is expected at chance.
- Eczema: a form of dermatitis or inflammation of the epidermis.
- ELISPOT assay: a capture assay in which cytokines produced by activated cells are trapped onto membranes coated with specific anti-cytokine antibodies.
- Endosome: a membrane-bound compartment allocated inside cells that is involved in intracellular transport.
- Enzyme Allergosorbent Test (EAST): enzyme solid phase immunoassay used for the determination of specific IgE antibodies in serum.
- Enzyme Linked Immunosorbent Assay (ELISA): a solid phase enzyme immunoassay developed in different formats and used for the quantitative measurement of antigens or antibodies.
- Epitope: an epitope is the part of an antigen that interacts with the antibody (or T cell receptor). Epitopes can be either conformational (i.e. determined by the 3D structure of the antigen) or linear (i.e. determined by a small stretch of contiguous amino acids).
- FasL: is a type II transmembrane protein that belongs to the TNF family.
- FASTA: the first widely used algorithm for database similarity searching. The program looks for optimal local alignments by scanning the sequence of a query protein and comparison with that of known allergens.
- Glycosylation: process by which sugar residues attach to proteins.
- GM-CSF: proteins secreted by macrophages, T cells, mast cells, endothelial cells and fibroblasts.
- Hapten: a small molecule that can induce a specific immune response when covalently linked to a carrier protein and not only by itself.
- IgE-abs: IgE-antibodies

- Immunoglobulins (Ig): serum antibodies, including IgA, IgD, IgE, IgG and IgM that are used by the immune system to identify and neutralize antigens. Each of the Ig is made up of two heavy chains and two light chains and has two antigen-binding sites.
- *In silico:* data generated and analysed using modelling and information technology approaches.
- *In vitro*: study in the laboratory usually involving serum, isolated organs, tissues, cells or cellular fractions.
- Inflammation: a series of reactions that bring cells and molecules of the immune system to sites of infection or damage. This appears as an increase in blood supply, increased vascular permeability, and increased transendothelial migration of leukocytes.
- Interferons: a group of molecules involved in signaling between cells of the immune system.
- Interleukins: a group of glycoproteins involved in signaling between cells of the immune system.
- Isoallergen: Isoallergens are defined (by the IUIS/WHO Allergen Nomenclature Sub-Committee) as molecules (from the same species) sharing similar size, identical biological function or feature ≥ 67 % amino acid sequence identity
- Leucotriene: naturally produced eicosanoid lipid mediators that may be responsible for the effects of an inflammatory response.
- Lymph nodes: an organ formed by many types of cells that is part of the lymphatic system.
- M cells: specialized epithelial cells of mucosal surfaces lining the respiratory and intestinal tracts. They participate in generating mucosal immune protection by sampling and delivering antigens to the underlying lymphoid tissue.
- Major allergens: allergens that are recognised by more than 50% of a population of individuals allergic to the food. The concept of major allergens relates only to the frequency of recognition by IgE antibodies, and it is not related to the severity of the clinical manifestations of an allergic reaction.
- Major histocompatibility complex: a genetic region found in most vertebrates that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances.
- Mimotope: a molecular sequence which mimics the epitopic region of a particular antigen, but which does not contain the specific amino acid sequence that comprises the epitope.
- Motif: An amino acid sequence motif can be described as a sequence of amino acids that defines a substructure in a protein that can be connected to function or to structural stability.
- NOD receptors: cytoplasmic proteins that may have a variety of functions in regulation of inflammatory and apoptotic responses.
- Pfam: database with a large collection of protein families, each represented by multiple sequence alignments, http://pfam.sanger.ac.uk/
- Presumed non-allergens: proteins with presumably low allergenic potential under normal conditions/exposure
- Primary sensitisation: the allergic reaction is elicited by the same allergen that induced the allergic sensitisation.
- Profiling: creation of patterns of the substances within a sample with the aid of analytical techniques, such as functional genomics, proteomics, or metabolomics. The identity of the compounds detectable within the pattern needs not to be previously recognized.
- Propensity scales: a propensity score is assigned to every amino acid, based on studies of their physico-chemical properties (hydrophobicity, normalized Van der Waals volume, polarity, polarizability, charge, surface tension, secondary structure and solvent accessibility)
- Proteomics: protein profiling using among others 2D-gel electrophoresis and mass spectrometry.
- Radio Allergosorbent Test (RAST): a solid-phase radioimmunoassay for detecting IgE antibody specific for a particular allergen.
- Ribonucleases: enzymes that catalyze the degradation of RNA
- Secondary allergy or cross-allergy: the allergic reaction is elicited by an allergen cross-reacting with the allergen that caused the sensitisation.
- Sequence alignment: a method to compare and represent similarities and differences between sequences of biomolecules.
- Skin-prick test: an allergy test that involves placing a small amount of suspected allergen to a scratch on the skin.

- T cell: Lymphocytes that differentiate primarily in the thymus and are central to the control and development of immune responses.
- T helper (Th1, Th2 and Th17): a sub-group of lymphocytes that play an important role in establishing and maximizing the immune response.
- Th (T-helper) cells: different types of T helper lymphocytes characterized by different cytokine production profiles: Th1 cells secrete IL-2, IFNγ… whereas Th2 cells secrete IL-4, IL-5…
- TGF (Transforming growth factors): a group of cytokines with the ability to promote fibroblast growth.
- TNF (tumor necrosis factor): a group of proinflammatory cytokines encoded within the MHC.
- Tolerance: a state of specific immunological unresponsiveness and therefore, inability to respond to antigenic stimulus.
- Toll-like receptors: a group of cell surface receptors of natural immunity that recognize molecules from pathogens.
- Transcription factor: proteins that bind to specific DNA sequences and control the transcription from DNA to RNA.