



Current and future methods for evaluating the allergenic potential of proteins: International workshop report 23–25 October 2007

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ABSTRACT

The International Life Science Institute's Health and Environmental Sciences Institute's Protein Allergenicity Technical Committee hosted an international workshop October 23–25, 2007, in Nice, France, to review and discuss existing and emerging methods and techniques for improving the current weight-of-evidence approach for evaluating the potential allergenicity of novel proteins. The workshop included over 40 international experts from government, industry, and academia. Their expertise represented a range of disciplines including immunology, chemistry, molecular biology, bioinformatics, and toxicology. Among participants, there was consensus that (1) current bioinformatic approaches are highly conservative; (2) advances in bioinformatics using structural comparisons of proteins may be helpful as the availability of structural data increases; (3) proteomics may prove useful for monitoring the natural variability in a plant's proteome and assessing the impact of biotechnology transformations on endogenous levels of allergens, but only when analytical techniques have been standardized and additional data are available on the natural variation of protein expression in non-transgenic bred plants; (4) basophil response assays are promising techniques, but need additional evaluation around specificity, sensitivity, and reproducibility; (5) additional research is required to develop and validate an animal model for the purpose of predicting protein allergenicity.

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1. Introduction

In the last 12 years, a number of foods produced using biotechnology have been introduced to the marketplace. These products have been carefully evaluated for their overall safety from an agronomic, environmental, performance, and equivalence perspective, and the safety of the newly expressed or novel protein. One aspect of the protein safety evaluation is the question concerning potential allergenicity. The allergy risk to consumers from crops enhanced through biotechnology may be placed into one of three categories. The first category that represents a risk to the allergic consumer is the transfer of a known allergen or cross-reacting allergen into a food crop. The second risk category is the potential

for increasing the allergenicity of a crop by increasing the expression of endogenous allergens. The last category involves expression of novel proteins that may become allergens *de novo*.

Approaches to identifying potential food allergens for purposes of safety assessment have been developed and modified over the past 15 years. They were first based on the safety assessment process outlined by Metcalfe et al. (1996) that followed a decision tree approach, and relied on gene source, protein homology, immunoglobulin E (IgE) reactivity and protein stability. Since that time, the assessment has subsequently evolved into the weight-of-evidence (WOE) approach as recommended by Codex (CAC, 2003). The current Codex recommendations recognize the limitations associated with the specific methods used as part of the WOE approach, and has encouraged that all suggested methods be scientifically supportable, and that additional work be done to clarify uncertainties associated with the methods.

As a consequence, laboratories around the world have been generating data and evaluating methods with the goal of providing

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improved analytical and predictive tools for evaluating protein allergenicity. In addition, several nations have or are currently supporting large research efforts to improve protein allergenicity prediction with multiple investigators. Examples include the Euro-Prevall project (www.europrevall.org), which is investigating the prevalence and distribution of food allergies throughout Europe in infants, children and adults as well as the role of the environment in determining different patterns of food allergy; and developing new diagnostic tools to provide better correlation of *in vitro* diagnostic results with clinical situations. Another example is the Korean Sera Bank project, which is assessing the prevalence and distribution of food allergy in Korea (Thomas et al., 2007). Moreover, the United States Environmental Protection Agency (EPA) together with the National Institute for Allergy and Disease/National Institute of Health (NIAD/NIH) has also sponsored two rounds of grants for projects involving the prediction of protein allergenicity. In particular, EPA's Office of Research and Development (ORD) has initiated a targeted research effort to address data gaps and improve the various recommended methods/endpoints for assessing the allergenic risks associated with plant incorporated pesticides (PIPs) that includes the development and evaluation of (1) animal allergy models; (2) targeted or specific serological assays; and (3) structure-activity relationship models.

The International Life Science Institute's (ILSI) Health and Environmental Sciences Institute's (HESI) Protein Allergenicity Technical Committee (PATC) has also contributed to the advancement of the science associated with characterizing the allergenic potential of novel proteins and biotechnology products. In particular, the PATC (www.hesiglobal.org) has sponsored workshops, ring studies, and research projects during the past eight years to inform and enhance the State of the Art regarding the WOE assessments conducted to assess the potential allergenicity of novel proteins prior to their commercial release. The most recent HESI workshop in this series was held October 23–25, 2007 in Nice, France. The objective of the workshop was to evaluate current and future methods and techniques for evaluating the allergenicity of novel proteins, and to consider and discuss their applicability in the broader context of hazard identification and risk assessment of protein allergenicity. The workshop brought together a wide variety of international experts from academia, industry and government, and from a variety disciplines including immunology, chemistry, molecular biology, bioinformatics, and toxicology. The purpose of this paper is to summarize the workshop discussions and consensus points on the utility and applicability of methods for assessing the potential allergenicity of novel proteins expressed in products produced through biotechnology, and to highlight the strengths and weaknesses of these methods in the context of improving the WOE assessment for evaluating protein allergenicity.

2. Bioinformatics methods for comparing novel proteins to known allergens

To address one of the allergy risks associated with the introduction of novel proteins into foods, a bioinformatics approach is followed as recommended by the Codex (CAC, 2003). The purpose is to identify proteins sufficiently similar to allergens to suspect potential cross-reactivity. If a protein of interest matches one of the sequences in the database, additional data (e.g., IgE sera screening studies) and scientific interpretation is required to make a decision regarding the likelihood that the query protein is an allergen (Goodman, 2006).

During the workshop, two main types of approaches were discussed. The first addressed the issues related to current bioinformatics methods, which are based on the comparison between

several linear sequences, and proposed several technical improvements to this process. Other new approaches are based on the protein conformational structure that may be useful for refining the WOE approach in the future, when proven predictive.

2.1. Linear sequence

Most bioinformatic tools are comparison tools that operate only at the linear amino acid sequence level and are adequate for directing subsequent studies when putative homologous matches are found. This bioinformatics approach utilizes, as its primary resource, protein allergen sequence databases. Today, many different allergen databases are developed and maintained. These databases differ in their content, accessibility, level of descriptive information, data (biological or molecular data, update date, number of sequences), degree of curation, and the presence of informatics applications for comparing the query novel sequence to public annotated sequences. The main issues with such databases are (1) how to merge all this information; (2) how to use it; (3) and how to track changes in the databases. In addition, to develop an environment for reliable analysis and data integration takes considerable time and resources (Gendel and Jenkins, 2006). A solution would be to exchange data through semantic web approaches. For instance, extensible markup language (XML), with a domain-specific ontology, could be used to facilitate the sharing of structured data across different information systems, particularly via the web.

The AllergenOnline database (www.allergenonline.com) is an allergen database that has been developed specifically for predicting the potential allergenicity of novel proteins by the Food Allergy Research and Resource Program (FARRP). AllergenOnline is a free, publicly available, well-curated, archived resource list of known and putative allergens and sequences that may be searched by FASTA3 for the safety assessment of novel proteins. Version 8.0 of the database (January, 2008), includes 1313 unique sequences that are clustered into 484 allergen groups based on species ($n = 230$) and sequence identities. Peer-reviewed scientific publications for evidence of allergenicity of the proteins are also available on-line. The search routines include both overall best alignment as well as sliding 80 amino acid window searches, consistent with the Codex recommendations (CAC, 2003). The database is updated annually by searching National Center for Biotechnology Information (NCBI) and International Union of Immunological Societies (IUIS), and by evaluating the candidate entries for evidence of allergenicity using a peer review panel of international food allergy experts. These experts assess whether proteins are true allergens or putative allergens by using predefined criteria, which are described on the website.

Considerable attention was given to the criteria that define a "significant homology" by which to evaluate the results of an allergen comparison (Silvanovich et al., 2006; Ladics et al., 2007). For example, the use of a 6 continuous, identical amino acid match to identify potential IgE epitopes in conjunction with a greater than 35% identity match over any 80 amino acid stretch as a threshold of concern has stimulated debate around their utility for predicting protein allergenicity (Hileman et al., 2002; Kleter and Peijnenber, 2002; Stadler and Stadler, 2003; Thomas et al., 2005a). Further, the general utility of short contiguous amino acid matches for predicting protein allergenicity has been strongly challenged (Silvanovich et al., 2006; Goodman et al., 2008). Therefore, although conservative [sequence searches for matches of >50% identity over 80 or greater amino acids may be more realistic (Goodman et al., 2008)] the >35% identity match over 80 or greater amino acids criteria was considered more relevant than the short contiguous identical amino acid (i.e., theoretical epitope) search based on data indicating protein cross-reactivity occurring

between Bet v 1 and vegetable proteins at approximately 40% protein identity (Scheurer et al., 1999).

In addition, Ladics et al. (2007) questioned the positive predictive value of the 35% or greater identity over an 80 amino acid sequence using a 'sliding window' algorithm (FAO/WHO, 2001). False positive and negative rates were evaluated using the 'sliding window' of 80 amino acids versus conventional FASTA analyses. Data indicated that a conventional FASTA analysis across the whole protein sequence produced fewer false positives and equivalent false negative rates compared to the 80 amino acid 'sliding window' FASTA search. In addition, conventional FASTA derived *E* scores, which reflect the potential random occurrence of aligned sequences, and which can be used to evaluate the significance of an observed alignment, were generally more significant (i.e., lower in value) than the sliding window derived *E* scores. These data also suggest that a conventional FASTA search provides more relevant identity to the query protein and better reflects functional similarities between proteins than a 'sliding window' search.

Moreover, Silvanovich et al. (2006) noted that, using the current 35% identity match over 80 or greater residues sliding FASTA window criteria, the same rate of false positives could be achieved using either random selected protein sequences or the same sequences after being subjected to 1000 rounds of sequence "shuffling". Therefore, rather than using a specific percent identity cutoff value, a methodology for determining an *E* score threshold was proposed. For instance, by applying the *E* score threshold methodology to 7695 corn protein sequences, a FASTA *E* score cutoff of 4.7×10^{-7} was determined. The cutoff was 100% effective at identifying known allergens, but was sufficiently conservative as to have a 95% false positive rate (i.e., only 1 out of 20 positives would reflect a true potential for cross-reactivity) such that no potential positive matches would be overlooked.

2.2. Conformational sequence

Workshop participants further discussed the benefits associated with creating ontology for allergens in an effort to harmonize the data and structural features in the available allergen databases. It was acknowledged that this would require a significant effort.

Greater importance and priority was given to assessing the utility of the structural database of allergenic proteins (SDAP) database (<http://fermi.utmb.edu/SDAP/>). SDAP is a web server that provides rapid, cross-referenced access to the sequences, structures and IgE epitopes of allergenic proteins. The SDAP core is a series of scripts that process the user queries, interrogate the database, perform various computations related to protein allergenic determinants and prepare the output internet pages. The database component of SDAP contains information about the allergen name, source, sequence, structure, IgE epitopes and literature references, as well as easy links to the major protein (PDB, SWISS-PROT/TrEMBL, PIR-ALN, NCBI Taxonomy Browser) and literature (PubMed, MEDLINE) on-line servers. The computational component in SDAP uses an original algorithm based on conserved properties of amino acid side chains to identify regions of known allergens similar to user-supplied peptides or selected from the SDAP database of IgE epitopes.

These types of bioinformatic tools may have the potential to rapidly determine a potential cross-reactivity between allergens and to screen novel proteins for the presence of IgE epitopes they may share with known allergens. However, major limitations to these methods suggest they are not yet predictive. In particular, there are relatively few allergens for which the crystal structure is known and the protein structure may be more related to the intrinsic function of the protein and less to the form in which the protein may sensitize a person or elicit an allergenic response. Therefore, further data are required to assess its utility in predict-

ing protein cross-reactivity. Participants recommended comparing the physico-chemical properties of discrete areas of allergenic proteins to known IgE epitopes, given that IgE epitopes may correlate with low 'Property Distance' values in designed peptide sequences. In particular, the identification of sequence motifs that could characterize protein allergenicity may be useful.

In conclusion, the consensus of the participants was that the bioinformatics techniques based on linear sequence comparisons could be improved by using more advanced tools, such as *E* score thresholds. In addition, to increase the power of the bioinformatics analyses, the determination of the degree of similarity between proteins and known allergens by using a structural database and appropriate comparison scripts may prove useful in the future.

3. Role of proteomics in the weight-of-evidence allergen assessment process

Participants agreed that another risk category, altering endogenous allergen levels, needs to be considered from the perspective of potentially sensitizing a larger portion of the population, as opposed to altering the reactivity of an already sensitized individual. Three approaches were envisioned to address this potential issue:

- (1) Allergen levels can be measured directly using techniques such as protein specific enzyme linked immunosorbent assays (ELISA). The main limitation of this approach is access to validated methods, reagents and standards.
- (2) Crop extracts could be screened for reactivity with sera from allergic patients with demonstrated and well-characterized sensitivity to the food of interest. The main limitation of this approach is access to sufficient quantities of well-characterized allergic subject serum (Thomas et al., 2007).
- (3) Alternatively, the relative allergen levels could be assessed via proteomic approaches (Cellini et al., 2004; Kuiper et al., 2003; Selgrade et al., 2003).

There was support among participants that proteomics approaches may be considered as complementary tools for characterizing potential changes in the composition of crops, including endogenous allergens. Several proteomics methods are already available for qualitative and quantitative analysis of plant proteome. The two-dimensional gel electrophoresis (2-DE) constitutes a rapid high resolution and powerful technique that is the most widely used approach to compare crop proteomes. It separates proteins according to their isoelectric point (pI) in the first dimension and molecular mass (M_r) in the second dimension. Coupled with mass spectrometry (MS), it also allows the detection and simultaneous quantification of several hundreds of individual proteins present in complex protein mixtures (Righetti et al., 2004). This approach can also identify peptides utilizing liquid chromatography coupled with tandem mass spectrometry and specialized analysis of digital images (<http://oilseedproteomics.missouri.edu>). Allergens can be observed as multiple spots after 2-DE gel electrophoresis due to heteromeric properties (e.g., soybean conglycinin). Biological and chemical post-translational modifications, due to differential glycosylation or partial deamidation, respectively, can also allow for observations of highly similar proteins, component peptides, and subunits.

The main challenge for routine application of proteomics techniques in safety assessment of transgenic crops is to validate and standardize methodologies to ensure its reproducibility and robustness, and to interpret the significance of the large quantity of data generated. Therefore, it is necessary to evaluate the natural variability of those allergens in non-transgenic crops, especially for

crops such as soybean that contain more than 20 known allergenic proteins, having different genetic backgrounds and grown in different environmental conditions (e.g., greenhouse vs. field, dry vs. wet climates, etc.). Preliminary data in soybeans illustrated this point by showing that major variations in protein profiles were observed between different soybean groups rather than among genotypes in the same group (Natarajan et al., 2005, 2006, 2007). In addition, although the ability to analytically quantify individual allergenic proteins using proteomics approaches is improving, it is not possible to correlate protein expression with biological relevance because of limited data on quantitative thresholds for sensitizing individuals to most allergens (Goodman et al., 2008).

The applicability of such proteomics approaches for the safety assessment of transgenic crops was evaluated in several projects (GMOCARE, 2008; Ruebelt et al., 2006). Laboratories around the world are also collecting proteomics data and making these data publicly available through publications (Natarajan et al., 2005, 2006, 2007) or via the internet (<http://oilseedproteomics.missouri.edu>). Limitations on the ability to quantify protein levels remain a drawback to this approach; however, improvements in separation techniques, protein preparations, and detection methods are evolving rapidly in this area. Label-free proteomics approaches were described in which protein quantification is performed within a mass spectrometer by either spectral counting or ion peak integration. Preliminary results suggested that this approach could provide the throughput and quantitative range to investigate seed phenotyping studies for prominent proteins, such as the major seed allergens.

Therefore, the consensus among the participants was that this approach is of interest and may be a direction for future research; however, a major priority in applying this approach to the safety assessment process involves determining the natural variation of proteins (e.g., allergens) in non-transgenic plant varieties grown under different environmental conditions.

4. In vitro methods: basophil activation assays

In vitro assays can be used to address the cross-reactivity risk to the allergic consumer, i.e., transfer of a known allergen or cross-reacting allergen into a food crop. While specific IgE is necessary to cause allergic disease, its presence in sera is not necessarily indicative that the patient is clinically allergic to the protein. The availability of serum from well-characterized allergic patients with true food allergy symptoms (i.e., IgE-mediated anaphylaxis) is limited. In many cases, ethical and practical considerations hamper the ability to collect sufficient samples to conduct studies that facilitate statistically meaningful results, particularly when the allergy of interest involves a minor allergen (i.e., less than 50% of the allergic population react to this specific allergen).

For predictive purposes, *in vitro* methods would be quite attractive, although no *in vitro* method is expected to reach the level of predictability that a human *in vivo* challenge study could provide. During the workshop, two presentations illustrated the utility and limitations of such methods by using basophils and Rat Basophilic Leukemia cell lines as examples for evaluating the allergenicity of novel proteins.

4.1. Basophil assay

Basophils are granulocytes originating from CD34+ stem cells and are similar to mast cells, as both mediate the release of histamine in response to IgE crosslinking. Basophils express the high-affinity IgE receptor FcεR1. When this receptor binds the IgE-antigen complex, basophils degranulate and release mediators (e.g., histamine, IL-4, IL-13, neutral proteases, lysosomal enzymes)

causing many of the clinical symptoms observed in allergenic responses. The use of basophils from clinically documented allergic patients was described in the diagnosis of allergic diseases to investigate the presence of specific IgE in the serum of patients. The first *in vitro* test developed was the histamine release test that requires a considerable amount of blood and subsequent isolation of basophils, and has a low sensitivity and specificity.

Here the use of a most recent technique based on flow cytometry, which uses specific antibodies to basophil markers and requires very little blood from the allergic patient, was described. This technique allows counting the proportion of cells expressing a molecular marker in a given population of cells. Basophil activation is measured by surface expression of the marker of degranulation CD63, or the marker of activation of basophils CD203c, which are rapidly up-regulated upon stimulation. Additional activation biomarkers have also been identified on basophils, i.e. CD13, CD164, and CD107a. They may further improve the flow cytometric study design using basophil activation parameters as biologically relevant test system endpoints for predicting allergenicity. It is interesting to note that there is a possibility to use basophils from donors who are atopic, but not food allergic. In this case, basophils are passively sensitized using serum from allergic patients and then challenged using the specific allergens.

Importantly, very few proteins, especially food proteins, have been evaluated with this technique and it should be validated before applying it to the regulatory safety evaluation of novel proteins. In addition, the inter-laboratory reproducibility of the assay should also be examined in detail. For instance, basophil integrity declines rapidly over a period of 8 hours and thus, it is difficult to set up a robust protocol with appropriate controls. Standardization, therefore, would initially be required to begin validating the assay.

4.2. Rat basophilic leukemia (RBL) assay

Specific IgE sera screening studies are employed to investigate protein cross-reactivity; however, they are non-functional immunochemical methods and cannot measure the biological reactivity of proteins. Therefore, an assay using RBL cells transfected with human FcγRI IgE receptor was developed in order to determine the ability of allergen extracts to induce a type I allergic reaction. The human FcεRI receptor binds IgE from the serum of allergic subjects, and subsequently activates the cells in an allergen-specific manner. The release of mediators such as β-hexosaminidase is used to measure the degranulation.

Ladics et al. (2008) evaluated the degranulation of three such cell lines expressing either the α-(RBL-hE1a-2B12 and RBL-30/25 cells) or α-, β-, and γ-subunits (RBL SX-38) of the human FcεRI by β-hexosaminidase release. Purified human IgE and serum-derived polyclonal IgE from peanut-allergic subjects following challenge with anti-IgE or peanut protein extract, respectively, were employed. Using purified human IgE mixed with anti-human IgE, robust degranulation was observed with all three cell lines. Nevertheless, there were not consistent degranulation results with a potent food allergen (i.e., peanuts). While some investigators have demonstrated good intra-laboratory sensitivity and reproducibility with the humanized RBL cell lines (Dibbern et al., 2003; Vogel et al., 2005; Kaul et al., 2007), others (Marchand et al., 2003; Takagi et al., 2003) have not been able to generate consistent and robust responses using allergic subject sera. Therefore, although RBL cells can be serially cultured and the method can be easily standardized, the utility of the transfected RBL assay for predicting the potential cross-reactivity of a novel biotechnology protein with a known allergen needs to be further investigated. In particular, studies that test other known allergens and well-characterized allergic subject sera are needed before it is used in

the support of safety assessments for biotechnology-derived products and subsequent public health decision-making.

In conclusion, the participants suggested that, while *in vitro* methods hold promise, further evaluation and validation are needed. Specificity, sensitivity, inter-laboratory reproducibility, precision, accuracy, and robustness should be established for each assay.

5. Animal models for predicting human allergenicity

Validated animal models may help to address another category of risk that involves expression of novel proteins that may become allergens *de novo*. Today, this is the only method that could assess this type of risk. However, human food allergy is very complex and includes several factors (e.g., exposure, genetic predisposition, etc.). This explains why the human population itself is not consistent in developing food allergies. Only (1–2%) of adults have true food allergies; not all people that have food allergies are allergic to the same foods; and some allergies may be “outgrown” or tolerance developed. Moreover, this is the reason why no animal models have been fully validated so far.

The “ideal” animal model has been described as (1) having a similar allergenic response as a human; (2) having a similar relative reactivity to proteins on an “allergenicity” scale; thus discriminating allergens from non-allergens; (3) eliciting a response following administering proteins by typical routes of exposure (e.g., oral, dermal, etc.); (4) not requiring adjuvant; (5) using a manageable number of animals; (6) producing results reasonably quickly (months); (7) being reproducible, specific, and sensitive; (8) relatively inexpensive; and (9) supporting multiple analytical endpoints. There is no animal model currently being evaluated that achieves even a majority of these criteria, and participants agreed that several validated models might be needed for assessing potential protein allergenicity with a high level of accuracy. For example, several different models are necessary to study the mechanistic basis of allergic responses, to evaluate therapeutic approaches, or to assess the allergenicity of novel proteins.

Canine models have real advantages since their symptomatology is similar to human response (Buchanan and Frick, 2002; Teuber et al., 2002). However, the difficulty of maintaining colonies, the lengthy testing timeframe, and public perception issues with their utilization limit their value.

Rodent models are attractive because they cover the majority of the listed criteria above. However, none of the rodent models have been validated to date (Ladics et al., 2003; Knippels and Penninks, 2003, 2005; McClain and Bannon, 2006). In particular, the HESI ring trial results (Thomas et al., 2005b), which included participation from 5 international laboratories that evaluated 3 allergens and 2 non-allergens tested were presented. In general, administration of test proteins was done via intraperitoneal injection, a route to maximize the opportunity for elicitation of immune and allergic responses. This route of exposure also avoids the possibility of tolerance induction associated with oral administration. The results highlighted the difficulty to discriminate between allergens and non-allergens. In particular, the purification and the characterization of the test material were considered to be critical as they were the main concerns in this study. The test proteins must be highly purified to avoid confounding results due to contaminants, such as lipopolysaccharides or other endotoxins.

A mouse model, using the BALB/c mice strain for evaluating potential allergenicity, was presented. The experimental approach was configured to provide maximum sensitivity for the purposes of allergenicity hazard identification (Dearman et al., 2003a and 2007). To this end, IgE-predisposed BALB/c mice were used. Exposure was systemic via intraperitoneal injection (absence of adju-

vant). At various times after the initiation of exposure IgG antibody responses were measured using ELISA and IgE antibody, which is the major effector of immediate-type allergic reactions, were detected using a homologous passive cutaneous anaphylaxis (PCA). Although only a relatively limited number of proteins have been examined (5 in total) in two different laboratories, these data suggest that the measurement of antibody responses in BALB/c mice may identify food allergens and distinguish them from those materials that apparently lack significant allergenic potential (Atherton et al., 2002; Dearman et al., 2003b, 2007; Kimber et al., 2003; Herouet-Guicheney et al., *in press*). However, other independent laboratories need to evaluate its reproducibility and additional proteins need to be tested in the model. A second promising mouse model using C3H/HeJ mice was presented in which oral exposure of protein extracts (peanut, Brazil nut, egg white, spinach, or turkey), in presence of adjuvant (cholera toxin), distinguished allergenic from non-allergenic foods (Bowman and Selgrade, 2008). Again, these results should be confirmed by other independent laboratories and by testing additional allergenic and non-allergenic proteins.

Although it is generally considered that one *in vivo* model cannot predict all aspects of the food allergy among human populations exposed to a novel allergen in the diet, a validated and robust animal model could be useful for identifying intrinsic hazard, or lack of it. Research is therefore continuing in the development of animal models to predict protein allergenicity (Birmingham et al., 2005; Gizzarelli et al., 2006). There was discussion whether different mouse strains need to be developed with different mutations, perhaps, in an effort to make the animals respond more consistently and in the same relative intensity to known allergens including non-allergenic proteins. Work presented from the US EPA raised additional questions involving focus on utilizing purified proteins (with the possibility that they are not in their “native” state). Participants also raised concerns regarding the testing of whole foods, food extracts, or the biotechnology food crop, by questioning the relevance of the food matrix, which represents real-world food consumption. It was further agreed that additional endpoints should also be explored, e.g. cytokine kinetics, surface markers on immune cells after sensitization of animals, etc., as well as the balance between the induction of tolerance versus sensitization.

6. Conclusions

A substantial amount of research has been published in recent years that further the science associated with evaluating the allergenicity of novel proteins. This workshop was undertaken with the goal of bringing forward the latest scientific methodologies that could help support the WOE approach for evaluating the allergenicity of biotechnology-derived products. The overall conclusion of this workshop is that the assessment of protein allergenicity remains a work in progress with validation of new testing methods being required to move them nearer to being formally integrated into WOE allergy assessments. There was, however, consensus on several points at the workshop: (1) current bioinformatic approaches are highly conservative, and thus may cull genes that may have proven extremely useful; (2) advances in bioinformatics using structural comparisons of proteins may be helpful as the availability of structural data increases; (3) proteomics may prove to be a useful tool for monitoring the natural variability in a plant's protein expression profile and, eventually, to view the impact of biotechnology transformations on endogenous levels of allergens; however, this can only occur once the analytical techniques are more standardized and more information on the natural variation of protein expression in non-transgenic plants is established; (4)

basophil response assays that use flow cytometry and transfected RBL cell lines are promising techniques, but need additional evaluation around specificity, sensitivity, and reproducibility; (5) additional research is required to develop and validate animal model(s) for the purpose of predicting protein allergenicity. Lastly, additional endeavors like EPA's Office of Research and Development (ORD) targeted research effort are needed to address data gaps, improve the various recommended methods/endpoints, and develop additional endpoints that have been 'validated' based on 'sound science' for assessing the allergenic risks associated with plant incorporated pesticides and other genetically modified organisms.

Conflict of interest statement

Karluss Thomas, Corinne Herouet-Guicheney, Gregory Ladics, Scott McClain, Laura Priville, and Mike Woolhiser declare that there are no conflicts of interest.

Susan MacIntosh has a regulatory consultancy with clients from several agricultural biotechnology companies, such as Bayer Crop Science, Pioneer Seed Co., and Syngenta Seeds.

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References

- Atherton, K.T., Dearman, R.J., Kimber, I., 2002. Protein allergenicity in mice: a potential approach for hazard identification. *Ann. NY Acad. Sci.* 964, 163–172.
- Birmingham, N., Gangur, V., Samineni, S., Navuluri, L., Kelly, C., 2005. Hazelnut allergy: evidence that hazelnut can directly elicit specific IgE antibody response via activating type 2 cytokines in mice. *Int. Arch. Allergy Immunol.* 137, 295–302.
- Bowman, C.C., Selgrade, M.K., 2008. Differences in allergenic potential of food extracts following oral exposure in mice reflect differences in digestibility: Potential approaches to safety assessment. *Toxicol. Sci.* 102, 100–109.
- Buchanan, B.B., Frick, O.L., 2002. The dog as a model for food allergy. *Ann. NY Acad. Sci.* 964, 173–183.
- Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H.V., Engel, K.H., Gatehouse, A.M., Karenlampi, S., Kok, E.J., Leguay, J.J., Lehesranta, S., Noteborn, H.P., Pedersen, J., Smith, M., 2004. Unintended effects and their detection in genetically modified crops. *Food Chem. Toxicol.* 42, 1089–1125.
- Codex Alimentarius Commission (CAC), 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy, 30 June–5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and Appendix IV, Annex on the assessment of possible allergenicity, 47–60.
- Dearman, R.J., Skinner, R.A., Herouet, C., Labay, K., Debruyne, E., Kimber, I., 2003a. Induction of IgE antibody responses by protein allergens: inter-laboratory comparisons. *Food Chem. Toxicol.* 41, 1509–1516.
- Dearman, R.J., Skinner, R.A., Herouet, C., Labay, K., Debruyne, E., Kimber, I., 2003b. Induction of IgE antibody responses by protein allergens: inter-laboratory comparisons. *Food Chem. Toxicol.* 41, 1509–1516.
- Dearman, R.J., Kimber, I., 2007. A mouse model for food allergy using intraperitoneal sensitization. *Methods* 41, 91–98.
- Dibbern Jr., D.A., Palmer, G.W., Williams, P.B., Bock, S.A., Dreskin, S.C., 2003. RBL cells expressing human FcεRI are a sensitive tool for exploring functional IgE-allergen interactions: studies with sera from peanut-sensitive patients. *J. Immunol. Methods* 274, 37–45.
- FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. January 22–25, Rome, Italy, 2001.
- Gendel, S.M., Jenkins, J.A., 2006. Allergen sequence databases. *Mol. Nutr. Food Res.* 50, 633–637.
- Gizzarelli, F., Corinti, S., Barletta, B., Iacovacci, P., Brunetto, B., Butteroni, C., Afferni, C., Onori, R., Miraglia, M., Panzini, G., Di Felice, G., Tinghino, R., 2006. Evaluation of allergenicity of genetically modified soybean protein extract in a murine model of oral allergen-specific sensitization. *Clin. Exp. Allergy* 36, 238–248.
- GMOCARE, 2002. New methodologies for assessing the potential of unintended effects in genetically modified food crops (GMOCARE). Available at <http://www.entransfood.nl/RTDprojects/GMOCARE/GMOCARE.html> (Accessed on 11.1.2008).
- Goodman, R.E., 2006. Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches. *Mol. Nutr. Food Res.* 50, 655–660.
- Goodman, R.E., Vieths, S., Sampson, H.A., Hill, D., Ebisawa, M., Taylor, S.L., van Ree, R., 2008. Allergenicity assessment of genetically modified crops – what makes sense? *Nature Biotechnol.* 26, 73–81.
- Herouet-Guicheney, C., Aldemir, H., Bars, R., de Barbeyrac, D., Kennel, P., Rouquié, D., Stahl, B.U., Kimber, I., Dearman, R.J., in press. Inter-laboratory comparisons of assessment of the allergenic potential of proteins in mice. *J. Appl. Tox.*
- Hileman, R.E., Silvanovich, A., Goodman, R.E., Rice, E.A., Holleschak, G., Astwood, J.D., Hefle, S.L., 2002. Bioinformatic methods of allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128, 280–291.
- Kaul, S., Lüttkopf, D., Kastner, B., Vogel, L., Hölz, G., Vieths, S., Hoffmann, A., 2007. Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin. Exp. Allergy* 37, 141–150.
- Kimber, I., Dearman, R.J., Penninks, A.H., Knippels, L.M.J., Buchanan, R.B., Hammerberg, B., Jackson, H.A., Helm, R.M., 2003. Assessment of protein allergenicity on the basis of immune reactivity: animal models. *Environ. Health Perspect.* 111, 1125–1130.
- Kleter, G.A., Peijnenbor, A.C.M., 2002. Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens. *Biomed. Central* 2, 1–11.
- Knippels, L.M., Penninks, A.H., 2003. Assessment of the allergic potential of food protein extracts and proteins on oral application using the Brown Norway Rat model. *Environ. Health Perspect.* 111, 233–238.
- Knippels, L.M., Penninks, A.H., 2005. Recent advances using rodent models for predicting human allergenicity. *Toxicol. Appl. Pharmacol.* 207, 157–160.
- Kuiper, H.A., Kok, E.J., Engel, K.H., 2003. Exploitation of molecular profiling techniques for GM food safety assessment. *Curr. Opin. Biotechnol.* 14, 238–243.
- Ladics, G.S., van Bilsen, J.H.M., Brouwer, H.M.H., Vogel, L., Vieths, S., Knippels, L.M.J., 2008. Assessment of three human FcεRI-transfected RBL cell lines for identifying IgE induced degranulation utilizing peanut-allergic patient sera and peanut protein extract. *Reg. Toxicol. Pharm.* 51, 288–294.
- Ladics, G.S., Holsapple, M.P., Astwood, J.D., Kimber, I., Knippels, L.M., Helm, R.M., Dong, W., 2003. Workshop overview: approaches to the assessment of the allergenic potential of food from genetically modified crops. *Toxicol. Sci.* 73, 8–16.
- Ladics, G.S., Bannon, G.A., Silvanovich, A., Cressman, R.F., 2007. Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. *Mol. Nutr. Food Res.* 51, 985–998.
- Marchand, F., Mecheri, S., Guilloux, L., Iannascoli, B., Weyer, A., Blank, U., 2003. Human serum IgE-mediated mast cell degranulation shows poor correlation to allergen-specific IgE content. *Allergy* 58, 1037–1043.
- McClain, S., Bannon, G.A., 2006. Animal models of food allergy: opportunities and barriers. *Curr. Allergy Asthma Rep.* 6, 141–144.
- Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.* 36 (5), 165–186.
- Natarajan, S., Xu, C., Caperna, T.J., Garrett, W.M., 2005. Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. *Ann. Biochem.* 342, 214–220.
- Natarajan, S.S., Xu, C., Bae, H., Caperna, T.J., Garrett, W.M., 2006. Characterization of storage proteins in wild (Glycine soja) and cultivated (Glycine max) soybean seeds using proteomic analysis. *J. Agric. Food Chem.* 54, 3114–3120.
- Natarajan, S., Xu, C., Bae, H., Bailey, B.A., Cregan, P., Caperna, T.J., Garrett, W.M., Luthria, D., 2007. Proteomic and genetic analysis of glycinin subunits of sixteen soybean genotypes. *Plant Physiol. Biochem.* 45, 436–444.
- Righetti, P.G., Campostrini, N., Pascali, J., Hamdan, M., Astner, H., 2004. Quantitative proteomics: a review of different methodologies. *Eur. J. Mass Spectrom.* (Chichester, Eng.) 10, 335–348.
- Ruebelt, M.C., Lipp, M., Reynolds, T.L., Schmuë, J.J., Astwood, J.D., DellaPenna, D., Engel, K.H., Jany, K.D., 2006. Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 3. Assessing unintended effects. *J. Agric. Food Chem.* 54, 2169–2177.
- Ruebelt, M.C., Leimgruber, N.K., Lipp, M., Reynolds, T.L., Nemeth, M.A., Astwood, J.D., Engel, K.H., Jany, K.D., 2006. Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 1. Assessing analytical validation. *J. Agric. Food Chem.* 54, 2154–2161.
- Scheurer, S., Son, D.Y., Boehm, M., Karamloo, F., Franke, S., Hoffmann, A., Hausteiner, D., Veiths, S., 1999. Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. *Mol. Immunol.* 36, 155–167.
- Selgrade, M.K., Kimber, I., Goldman, L., Germolec, D.R., 2003. Assessment of allergenic potential of genetically modified foods: an agenda for future research. *Environ. Health Perspect.* 111, 1140–1141.
- Silvanovich, A., Nemeth, M.A., Song, P., Herman, R., Tagliani, L., Bannon, G.A., 2006. The value of short amino acid sequence matches for prediction of protein allergenicity. *Toxicol. Sci.* 90, 252–258.
- Stadler, M.B., Stadler, B.M., 2003. Allergenicity prediction by protein sequence. *FASEB J.* 17, 1141–1143.

- Takagi, K., Nakamura, R., Teshima, R., Sawada, J., 2003. Application of human Fc ϵ RI α -chain-transfected RBL-2H3 cells for estimation of active serum IgE. *Biol. Pharm. Bull.* 26, 252–255.
- Thomas, K., Bannon, G., Hefle, S., Herouet, C., Holsapple, M., Ladics, G., MacIntosh, S., Privalle L., 2005a. In silico methods for evaluating human allergenicity of novel proteins: International Bioinformatics Workshop Meeting Report: Mallorca, Spain, February 23–24. *Toxicol. Sci.* 88, 307–310.
- Thomas, K., Herouet, C., Bannon, G., Ladics, G.S., MacIntosh, S., Privalle, L., Woolhiser, M., 2005b. Evaluation of mouse models for assessing the allergenic potential of proteins. *Toxicologist* 84 (Suppl.), 1307. Abstract.
- Thomas, K., Bannon, G., Herouet-Guicheney, C., Ladics, G., Lee, L., Lee, S., Privalle, L., Ballmer-Weber, B., Vieths, S., 2007. The utility of an international sera bank for use in evaluating the potential human allergenicity of novel proteins: workshop report. *Toxicol. Sci.* 97, 27–31.
- Teuber, S.S., del Val, G., Morigasaki, S., Jung, H.R., Eisele, P.H., Frick, O.L., Buchanan, B.B., 2002. The atopic dog as a model of peanut and tree nut allergy. *J. Allergy Clin. Immunol.* 110, 921–927.
- Vogel, L., Lüttkopf, D., Hatahet, L., Haustein, D., Vieths, S., 2005. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 60, 1021–1028.