FOLLOW-UP OF THE WORKING GROUP MEETING SELF TASK ON ALLERGENICITY ASSESSMENT HELD ON 24 SEPTEMBER 2007 (BRUSSELS)

AGENDA

PARTICIPANTS

GMO Panel and Working Group (WG) members:
Rob Aalberse, Karine Hoffmann-Sommergruber, Gijs Kleter, Martinus Lovik, Gabriel Peltre, Jean-Marie Saint-Rémy, Willem Seinen, Daniel Soeria-Atmadja, Jean-Michel Wal (Chair) and John Warner.

EFSA:
Suzy Renckens and Ellen Van Haver.

APOLOGIES:
Christer Andersson, Philippe Eigenmann, Ralf Einspanier and Clare Mills[1].

1. WELCOME AND APOLOGIES FOR ABSENCE

The Chairman opened the meeting and welcomed all. Apologies for absence were received from some working group (WG) members as mentioned above.

[1] Clare participated to the discussions of Chapter 3 by teleconference.

2. DECLARATIONS OF INTERESTS

Those Working Group (WG) members that have not yet updated their annual declaration of interest (ADoI) will receive an e-mail from EFSA to remind them to update their ADoI.

3. MINUTES OF 16 JULY MEETING – FOLLOW-UP

Comments on and follow-up of the minutes of 16 July:

- It needs to be emphasised that besides gastro-intestinal sensitisation, sensitisation can also occur via the non-gastro-intestinal tract, such as via inhalation and the skin.
- Allergy in animals (currently covered by Chapter 1.8): statements on allergy in animals (such as the statement that piglets are immune-competent) might need to be checked by an expert in the field. **Ralf Einspanier** will be asked whether he can check this issue and whether other experts need to be approached. Professor Chris Stokes from the Bristol Veterinary School has a lot of expertise with allergy in animals (see also below under Chapter 1).
- As the draft document is besides IgE mediated reactions also covering non-IgE immune responses to foods, it would be useful to explain the role of the different subclasses of IgG and their relationships with allergy, acknowledging that this is a contentious area (as Codex is for instance only focussing on IgE-mediated reactions). **Rob** will write a paragraph in Chapter 1 and **John** will address the possible clinical impacts of the different antibodies involved in the Clinical Chapter (see also below under Chapters 1 and 2).
- **Jean-Michel** will compile the different responses from the GMO Panel that have been used to address Member States comments on GMO applications that are related to adjuvanticity of Cry-proteins. This compilation will be useful to address the comments from Norway and to prepare a possible meeting with Norwegian experts by the end of this year.

4. DISCUSSION OF THE DIFFERENT CHAPTERS

As a follow-up to the last WG meeting of 16 July, it was the aim to discuss those chapters that were not discussed on 16 July, as well as the chapters that have been updated since last meeting, focussing on the new paragraphs.

Chapter 1 (General Intro):

- Rob suggested to write a text on epitopes for Chapter 1.3 as the definitions of epitopes used throughout the document are slightly different. In addition, there is no clear cut between linear and conformational epitopes and the impact of post-translational modifications. Epitopes within a protein molecule should be clearly distinguished from isolated peptide fragments. References to epitopes along the document need to be consistent with this text.
- As the draft document is besides IgE mediated reactions also covering non-IgE immune responses to foods, it would be useful to explain the role of the different subclasses of IgG and their relationships with allergy, acknowledging that this is a contentious area (as Codex is for instance only focussing on IgE-mediated reactions). **Rob** will write a short paragraph on this issue in Chapter 1.

- Allergy in animals (currently covered by Chapter 1.8): statements on allergy in animals (such as the statement that piglets are immune-competent) might need to be checked by an expert in the field. **Ralf Einspanier** will be asked whether he can check this issue and whether other experts need to be approached. Professor Chris Stokes from the Bristol Veterinary School has a lot of expertise with allergy in animals.

Martinus presented new text on **adjuvanticity** (Chapter 1.4) and the following issues need to be further elaborated (**Martinus**):

- Adjuvanticity of diesel particles has not been unequivocally demonstrated. Gabriel informed about his own research on diesel particles in lab animals, showing high adjuvanticity of various fractions. Martinus mentioned that ultra-fine particles, as chemically inert or reactive particles, can be adjuvants.
- A distinction will be made between compounds that have a direct and indirect adjuvant activity, including indirect effects through stimulation of uptake of allergens, e.g. by saponins in foods. Substances promoting gut permeability may stimulate allergy similar to Th2 adjuvants.
- It needs to be highlighted that adjuvanticity can be beneficial (Th1 response can decrease the risk of allergenicity) or negative (sensitising potential in the presence of adjuvants).
- Related topics, such as immune response modifier, and breaking of self-tolerance and induction of autoimmunity should not be addressed (as this would widen the scope).
- We need to think about recommendations on how adjuvanticity should be assessed. There is no definite test for the prediction of adjuvanticity as there is no definite test for the prediction of allergenicity.
- A specific Th2-adjuvant potential identified in a mouse-model might be regarded as a hazard or a warning signal and a Th2-response might then require further assessment. This issue can be further addressed as a recommendation/perspective (in Chapter 6 in the context of the whole GM plant, or in Chapter 7 on animal models).
- Th2-sensitising effects in mouse do however not necessarily induce an effect in man. Human exposure studies or post-market monitoring might therefore be needed.

Chapter 2 on Clinical aspects

John and Jean-Marie introduced shortly the Chapter on clinical aspects.

- The immunological vs. the clinical reactivity needs to be more explicit in the Introductory paragraph (**John**).
- As the draft document is besides IgE mediated reactions also covering non-IgE immune responses to foods, it would be useful to explain the role of the different subclasses of IgG and their relationships with allergy, acknowledging that this is a contentious area (as Codex is for instance only focussing on IgE-mediated reactions). **John** will address the possible clinical impacts of the different antibodies involved in the Clinical Chapter.
- The mechanisms of non-IgE mediated reactions are considered to be addressed explicitly enough in the text.

The following recommendations were shortly discussed:

- More sera from patients are needed but they need also to be well-characterised. Statistical calculations have been done showing that 60-70 well-characterised sera are needed based on variability. Since this might not be feasible, the WG has to consider the reliability of studies performed with a lower number of sera.

- Regarding post-market surveillance, descriptions of reporting systems performed in France, Norway, Germany, Switzerland and Austria can be provided.
- Infants are more susceptible towards allergenic reactions as their gastro-intestinal tract differs from adults. A specific assessment for children might therefore be recommended. It needs however to be discussed how this specific pre-market assessment needs to be performed. It might for instance be recommended that more research is needed on young animal models.

Chapter 3: Structural aspects

Clare (by teleconference) and Karin presented their latest version of Chapter 3.

The following issues were discussed and need to be further elaborated in the text (**Karin/Clare**):
- Chapter 3 needs to be more applicable to GM.
- A new paragraph will be included on regulating deliberately (by genetic modification) or naturally the amount of specific proteins in plants. This would accommodate the section on transgenic plants down-regulating allergenicity.
- Another sub-section on post-harvest modifications will be added, besides those on biosynthesis in the plant including post-translational modifications.
- Would certain scaffolds lead to sensitisation? The example was raised how to assess a protein that belongs to a protein family which comprises numerous common allergens, e.g. the cupin family, while there is no or low sequence homology, and consequently unlikelihood for cross reactivity, with known allergens. The potential of this protein for de-novo sensitisation remains the main concern which needs to be further checked, particularly if it is stable towards digestion. Testing in an animal model is however not yet a requirement.
- Additional issues may be considered in a multi-step *in silico* analysis, such as clusters of homology, conservation of potential epitopes, ... T cell epitopes may also be taken into account.
- It also needs to be defined what is the meaning of low sequence homology. 35% alignment using a 80-amino acid sliding window might indicate cross-reactivity. Below 25%, alignment might in many cases not be relevant.
- The relevance of the 3-D structure for predicting the allergenicity of proteins was discussed. The sequence homology using an 80-amino acid sliding window does not tell anything about the 3-D structure. Sequence similarity within a particular important domain might be more relevant.
- Another criterion to take into account is in which part of the plant the protein is expressed. For instance, many cupins do not occur in the edible part of the plant.
- A paragraph needs to be added to explain how to assign a new protein to a certain protein family. The Pfam database is used for this purpose. It needs however to be clarified that this is a general database for all proteins, but not for allergens (in contradiction to the allergen databases mentioned in Chapter 4). The issue whether protein folding might help in assigning a new protein to a protein family was debated.
- An introductory paragraph to Chapter 3 needs to explain the connection between Chapters 3 and 4. Chapter 3 addresses the structural features of a protein, whereas Chapter 4 provides details how to assess the sequence homology
- The issue on digestibility needs to be further elaborated (Gijs, Chapter 5).

Chapter 4: Bioinformatics for the risk assessment of GM foods as regards potential allergenicity

The Bioinformatics' Chapter was presented by Daniel.

- With bioinformatics, cross-reactivity rather than the sensitising potential is looked at.
- The sequence homology based on the 6 or 8 contiguous amino acids was discussed. Matches of 6 amino acids are not specific enough to perform serum screenings, but also identical stretches of 8 amino acids do not necessarily mean the identification of potential epitopes. It was concluded that homology searches based on 6 contiguous amino acids should not be performed. In a publication of Kleter and Peijnenburg[2], methods for epitope prediction are combined, by further screening the positive outcomes of the sequence homology with a minimal length of six amino acids for the presence of potential linear IgE-epitopes (35% homology in a sliding window of 80 amino acids). The question was however raised whether this multi-step approach should be systematically performed and what to do in the case of positive results at the different stages.
- The FASTA approach appears to be better than the linear sliding window. There was some discussion with regard to the minimum threshold level for FASTA. Recommendations should be made in order to pick up potential cross-reactivity with sufficient sensitivity and acceptable specificity. The level of false positives that is acceptable needs to be agreed upon. This might however be a regulatory decision and not a scientific question. 35% is the threshold currently accepted. See also the comments provided by Rob attached to these minutes.
- The question was raised which databases need to be mentioned in Chapter 4.4, and whether some of them deserve to be recommended. It should be explained why particular databases are mentioned and that these are examples. To select the most suitable database, we need first to decide on what kind of procedure we are going to recommend and whether the search should be conducted on all the proteins or whether some of the proteins could be excluded because of a low importance with regard to allergenicity.
- As there are many databases and algorithms offered by websites, uniformization should be recommended.
- The issue was raised whether it would be possible to add information on the estimates of the sensitivity/specificity of the different computational methods as described under Chapter 4.7. It will however be difficult to compare the different databases because they have been validated with different datasets with different underlying algorithms and methods.

Chapter 6 (In vitro analysis for potential allergenicity testing of whole GM plants)

The aim of Chapter 6 is to cover *in vitro* analysis of the whole GM plant and to analyse possible modifications in its intrinsic allergenicity due to unintended effects, whereas Chapter 5 addresses *in vitro* methods for the assessment of the allergenicity of newly expressed proteins. Chapter 6 particularly concerns plants that are known to be food allergens. It is focussed towards the analysis of the allergen repertoire of the GM plant as compared with that of the conventional one in order to assess whether some endogenous allergens may be over-expressed after the genetic modification. Attention needs to be paid to the natural variability of proteins. The study on whether the whole GM crop is more allergenic than the non-GM crop should then be conducted both from a qualitative and quantitative point of view.

The following gaps were identified and need to be addressed in the chapter (**Gabriel**):

- Extraction of proteins and sample preparation (e.g. soluble/insoluble proteins).
- Separation then identification of proteins/allergens (e.g. proteomic analysis).
- Quantitative determinations methods, (RAST/EAST and inhibition assays).

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[2] Kleter and Peijnenburg (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. BMC Structural Biology 2002, 2:8.

- Profiling techniques, including glycomics, should be discussed with a careful attention to their relevance, appropriateness and validation obtained so far. Post-translational modifications of proteins as expressed in the plant need to be covered. Quantitative PCR of transcripts (transcriptomics) may also be an alternative sensitive method.
- The analysis of specific allergens in the whole crop should be carried out in analogy with the compositional analysis of the GM compared with the non-GM crop. The total spectrum of allergens, but also the glycosylation pattern needs to be looked at.
The difficulty of these methods is however that the outcome has to be interpreted correctly, and that the natural variation when comparing the non-GM with the GM crop needs to be taken into account. **Karin** will address this issue.
- Availability of sufficient number and volumes of sera in the case an allergen is expressed.
- Chapter 5 and 6 can cross-reference each other for methods that are relevant for both chapters (for instance ELISA, Western blot, proteomics).
- Micro-arrays and omics-technologies will be reviewed by **Gijs**.

5. DATE AND PLACE OF FUTURE MEETINGS

The next meeting is scheduled for 23 January 2008 in London (venue: Medical Research Council).

6. ACTION ITEMS (BY 10 JANUARY 2008)

The WG members are requested to provide i) the completed and revised version of their own chapter with regards to the comments made during the WG meeting and ii) their written comments on the other chapters in advance of the meeting in order to facilitate the discussions at the next meeting. Chapter 8, which needs to address the integration of the different approaches, will be discussed. The WG members are therefore asked to reflect on perspectives and recommendations and to draft corresponding text at the end of each chapter.

We will need to distinguish between three kinds of recommendations:
- Guidance to applicants: how to improve current practices
- Research gaps: recommendations for further research
- Recommendations to risk managers, for instance the need for databases and serum banks.

Rob provided some written recommendations regarding the assessment of the risk of potential cross-reactivity, which are attached to these minutes and will be discussed at the next meeting.

Annex: Recommendations regarding the assessment of the risk of potential cross-reactivity (Rob Aalberse, 24 September 2007)

1. The amino acid sequence of a significant number of "minor" allergens is not yet in the database. These will thus be invisible at the important in-silico checkpoint. It is important to apply proteomics to get better coverage of the allergen repertoire, particularly for food allergens.
2. The effects of post-translational modification should be fully incorporated into the risk assessment. This implies (1) removal from the allergen database of amino acids sequences of proteins for which IgE binding is completely due to post-translational attachment of glycans; (2) addition of information on posttranslational modifications that significantly affects IgE binding.
3. It is crucial that post-translational modification is investigated in the final host.
4. The current in-silico procedures for establishing potential cross-reactivity are imperfect, partially because reliable quantitative information on cross-reactivity is insufficient. A decision has to be made on the acceptability of false-negative hits (sensitivity versus specificity). A too strict adherence to sensitivity will result in an unreasonable number of false-positive hits, without completely avoiding all cross-reactivity risk.
5. The sensitivity/specificity profile for full identity over 6 (or 8) contiguous amino acids is poor. This analysis should not be advocated.
6. Partial identity of either a sliding window of 80 amino acids and/or full-length proteins is the preferred approach. The sliding window approach may be more appropriate if the target protein has (or is predicted to have) a multi-domain structure, as a single domain with similarity to a known allergen may escape detection if inserted into an otherwise non-allergenic protein. The 35% identity cut-off level is considered to be conservative and the use of a 50% identity cut-off has been suggested, but significant cross-reactivity may occur below 50% identity.