



Analytical criteria for performance characteristics of IgE binding methods for evaluating safety of biotech food products

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ABSTRACT

There is detailed guidance on how to perform bioinformatic analyses and enzymatic degradation studies for genetically modified crops under consideration for approval by regulatory agencies; however, there is no consensus in the scientific community on the details of how to perform IgE serum studies. IgE serum studies are an important safety component to acceptance of genetically modified crops when the introduced protein is novel, the introduced protein is similar to known allergens, or the crop is allergenic. In this manuscript, we describe the characteristics of the reagents, validation of assay performance, and data analysis necessary to optimize the information obtained from serum testing of novel proteins and genetically modified (GM) crops and to make results more accurate and comparable between different investigations.

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1. Introduction

The primary allergy risk to consumers from genetically modified crops may be placed into one of three categories. The first that represents the highest risk to the allergic consumer is the transfer of a known allergen or IgE cross-reacting protein into a food crop. The second category, which represents a relatively low risk to the allergic consumer, is the potential for an increase in the endogenous allergenicity of an already allergenic crop. This category combines the inherent risk from allergenic proteins that are native to the plant plus the additional risk that may arise due to the genetic modification (Goodman et al., 2005; Sten et al., 2004). The last category involves expression of novel proteins that may become allergens in man and generally represents a relatively low risk to the consumer, although this possibility has received attention of late (Lehrer and Bannon, 2005). In order to mitigate the three categories of potential allergy risk associated with biotech crops, all genes introduced into food crops undergo a series of tests that includes determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein

and known allergens; its susceptibility to enzymatic degradation; and serum screens using documented sera from allergic individuals if the protein is similar to known allergens or comes from an allergenic source (FAO/WHO, 2003). While there is detailed guidance on how to perform bioinformatic analyses (Thomas et al., 2005) and enzymatic degradation studies (Thomas et al., 2004), there is no consensus in the scientific community on the details of how to perform serum studies for regulatory agencies. IgE binding assays such as radioallergosorbent tests (RAST) (Sampson and Albergo, 1984), enzyme-linked immunosorbent assays (ELISA) (Yunginger and Adolphson, 1992) or immunoblotting (Burks and Fuchs, 1995) assays are available for this type of testing. Examples utilizing each of these different techniques to determine the IgE binding capacity of transgenic vs. non-transgenic foods and biotech proteins have appeared in the literature (Burks and Fuchs, 1995; Kim et al., 2006; Batista et al., 2005; Hoff et al., 2007).

All these assays use IgE in serum from sensitized individuals. Sensitized individuals should be allergic to the food from which the transferred gene was derived as shown by a convincing clinical history (Burks et al., 1988) or positive responses in double-blind, placebo controlled food challenges (Thomas et al., 2004; Bock et al., 1988). In this chapter, we describe the characteristics of the reagents, validation of assay performance, and data analysis necessary to optimize the information obtained from serum testing of novel proteins and genetically modified (GM) crops and to make results more accurate and comparable between different investigations.

Abbreviations: CAP-PEIA, capsulated hydrophilic carrier polymer fluoroenzyme immunoassay; CCDs, cross-reactive carbohydrate determinants; ELISA, enzyme-linked immunosorbent assays; GM, genetically modified; LOD, limit of detection; LOQ, limit of quantitation.

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2. Reagents

2.1. Sera

The quality of the IgE used in IgE binding assays is critical to the usefulness of any data that is generated from such assays. Therefore, serum donors need to be selected with relevant, clinically proven allergies based on a positive food challenge, ideally Double-Blind Placebo-Controlled Food Challenge (DBPCFC), to a specific food or allergen, or a documented convincing case history of anaphylactic reactions to that particular food or allergen (Thomas et al., 2004; Bock et al., 1988; van Ree et al., 2006; Burks, 2000). The IgE in the sera should be characterized by determining the total amount of IgE present and the amount of IgE that is specific to a food extract or to a specific allergen. This is usually accomplished by using capsulated hydrolytic carrier polymer fluoroenzyme immunoassay (CAP-FEIA; Phadia, Uppsala, Sweden). CAP-FEIA is a detection method, which has been used to assess food-specific IgE concentrations that are indicative of a patient being sensitized to an allergenic food (van Ree et al., 2006; Sampson, 2001). Independent of the analytical method chosen to determine specific IgE, the method needs to be accurate and reproducible. Sera of subjects with clinically proven food allergy should be excluded from the study if the amount of specific IgE does not allow a quantitative read-out in accordance to the validated limit of quantification of the method.

The importance of using well characterized, clinically documented patient sera for sera screens of GM proteins and food crops is best demonstrated with the case of IgE directed against carbohydrate determinants. About 20% or more of allergy patients generate specific anti-glycan IgE. Even though antibody-binding glycoproteins are widespread in pollens, foods and insect venoms, cross-reactive carbohydrate determinants (CCDs) do not appear to cause clinical symptoms in most, if not all patients. When IgE binding is solely due to CCDs, a glycoprotein allergen thus can be rated as a clinically irrelevant allergen (Altmann, 2007).

2.2. Target proteins

Target proteins used in IgE ELISA assays, both as test substances or control proteins, should be of high purity and sufficiently characterized to ensure their identity. However, there may be cases where this level of purity is unattainable. In these cases contaminating proteins should be identified and steps taken to ensure that the contaminating proteins do not interfere with the ELISA assay. For example, if the target protein was purified from a natural food source, the absence of IgE-reactivity to non-target protein impurities should be proven. The preferred source of the protein of interest is that they be isolated directly from the plant or food from which they originated. However, many times the target protein has been produced in a heterologous system such as *E. coli* because it is impossible to obtain sufficient quantities of the protein from the plant or food source. This is an acceptable practice as long as the heterologously produced protein is equivalent to the protein as it exists in the plant or food source. Equivalence between proteins expressed in different systems is typically established through a series of studies that compares the molecular weight, glycosylation status, protein folding, immunoreactivity, and functional activity of the proteins. If equivalence is established the heterologously produced protein can be used with confidence in *in vitro* assays.

2.3. Protein extracts

Protein extracts are prepared from finely ground powders of the source material (grain, food). Care should be taken to ensure that the source material has been verified as to its identity. This can

be accomplished by utilizing an event specific polymerase chain reaction assay or through chain of custody records. The finely ground powders are usually defatted first and then extracted while shaking in an aqueous buffer. Extracts are typically clarified by centrifugation and passed through a filter to remove particulates. If multiple independent extractions of a single sample are made then care should be taken to demonstrate that the independent extractions are comparable with respect to total protein content and protein constitution. The total protein concentration of the extracts can be determined by commercially available, ready to use kits and SDS-PAGE analysis can be performed to determine that each extract contains the same major proteins. Moreover, immunoblot analysis with specific sera can be done if the presence of contaminating proteins needs to be verified or excluded. Care should be taken to ensure that the protein extracts retain their immunological activity and that protein degradation due to extraction conditions or endogenous enzymatic activity is avoided. Finally, the stability of the protein in the aqueous extracts should be checked to ensure that storage does not lead to loss of specific protein components or immunological protein reactivity. If storage conditions affect sample stability, each sample or sample extract should be treated completely identical prior to analysis with regard to storage conditions including storage time and thawing. Confirming the identity of the source material and establishing the quality, reproducibility and stability of the extracts, provides confidence that differences in IgE binding are not due to artifacts of extract preparation and storage.

3. Assay requirements

There are numerous types of assays available for determining the amount of IgE bound to a specific protein or protein extract. The two most commonly used methods for determining the allergenicity of biotech proteins and GM crops are the IgE-immunoblot analysis and the ELISA assays. The immunoblot analysis is useful for determining the qualitative nature of IgE binding but it is difficult to quantify and can be prone to non-specific binding, especially when attempting to detect extremely low levels of IgE binding (Hoff et al., 2007). Moreover, the IgE-immunoblotting analysis includes a number of manually operated steps (electrophoresis in polyacrylamide gels, protein blotting, cutting and immunodetection of blotting strips) that require handling by well trained personnel and which are still prone to large assay-to-assay variation. In contrast, the ELISA assay is comparatively easy to handle, quantifiable, highly sensitive, and can be validated and standardized using specific criteria that can be reproduced in appropriately equipped laboratories with trained personnel. Nonetheless, the specificity of ELISA methods needs to be verified as well. The quantitative characteristics, standardization capabilities and ease of handling make it the preferred method for determining the IgE binding capacity of biotech proteins and GM crops. Because of these advantages we will use the ELISA assay to illustrate the method requirements that are used in a regulatory setting for determination of the IgE binding capacity of proteins.

3.1. IgE standard curve

For investigation of IgE binding in a quantitative context, a robust calibration curve needs to be established. By introducing an IgE standard curve into the test format, the performance of each of the test runs can be monitored and the validity of test results scrutinized. Therefore, data generated from IgE binding assays should be based on the amount of IgE that binds to each test extract or test protein. IgE binding can be quantified by use of an allergen or extract specific IgE standard curve and expressed as

ng IgE per ml of serum. The standard curve is created by loading serial dilutions of a serum that contains a known amount of IgE into wells coated with reference allergens or extracts. An example of a soy extract specific IgE standard curve is shown in Fig. 1A. In this example, serum from a soy allergic patient (PEI 163) was measured by CAP-FEIA to be 36 kU/l and then expressed as ng/ml by using a conversion factor of 2.4 ng per 1 U. The standard curves were generated with serial 4-fold dilutions of PEI 163 in an incubation buffer and loaded between 21.6 and 0.021 ng/ml of soy-specific IgE into wells coated with an internal reference soy extract. The amount of IgE binding to the soy extract was detected after subsequent incubation steps with biotinylated goat anti-human IgE antibodies and horseradish peroxidase conjugated streptavidin by measuring the optical density (OD) at 450 nm wavelength of the peroxidase mediated coloring of each well. One drawback of this method is that large quantities of a single, well characterized, patient's sera may be unavailable. Alternatively, a serum pool may be employed. In cases where appropriate allergen specific human sera are in insufficient quantities, a human myeloma IgE may be used to construct a standard curve independent from the target protein. Such human myeloma standard IgE can be adsorbed directly onto the well surface and then detected by the specific anti-IgE antibodies. However, as shown in Fig. 1B, the reproducibility of a standard curve and thus assay-to assay precision using myeloma IgE for adjusting appropriate ELISA sensitivity may be not as good as using single, well characterized patient sera specifically bound to the adsorbed target protein. The variance we observed in the myeloma IgE standard curve could be due to a variety of technical factors including difficulty in reproducibly diluting extremely concentrated solutions down to appropriate levels, day-to-day variations in myeloma IgE binding efficiencies to ELISA plates, and lot-to-lot variability of the myeloma IgE.

Another alternative if sufficient allergen specific human sera are unavailable is to construct a mouse/human chimeric monoclonal IgE antibody by recombinant DNA techniques from a mouse monoclonal antibody directed to a specific allergen. Schuurman et al. (1997) constructed a chimeric IgE antibody to Der p 2 a major mite allergen. This chimera (hIgE-Dp2A) had the binding characteristics of the original mouse monoclonal antibody 2B12, whereas the Fc domain is derived from human IgG. More precisely, this chimeric IgE consisted of the heavy-chain variable (VH) domains and the complete light chains both cloned from the mouse hybridoma, whereas the constant domains of the heavy-chain are of human

origin. Because all IgE in this preparation is allergen specific, it is useful as a reference reagent and enables the calculation of the amount of allergen specific IgE in absolute IgE mass units (Schuurman et al., 1997).

3.2. Intra- and inter-assay precision

For correct interpretation of quantitative data in terms of significant differences of IgE binding to the target protein vs. signal differences due to assay variability, intra- and inter-assay precision need to be determined. The intra-assay precision describes the precision of quantitative determination that can be achieved independently to the position of the sample in the ELISA plate whereas the inter-assay precision refers to the variability of the ELISA assay established by multiple individuals performing the assay on different days and determining the variability inherent to the performance of the assay by different individuals.

3.3. Limit of detection (LOD)

Limit of detection (LOD) should be determined for each IgE binding ELISA. The LOD is usually defined as the minimum amount of IgE (ng/ml) that would result in a signal large enough to be discriminated from a blank sample. According to the bell-shaped distribution of results from repetitive analysis, half of the results at the LOD would be found as true positive results whereas the other half would be interpreted as a negative result. The LOD is typically calculated by analyzing aliquots of extracts from non-protein of interest (POI) plant tissues or of non-allergenic proteins at a dilution where matrix effects would not be expected to interfere with the analysis. In practice, the LOD for the IgE binding assay usually is the concentration equivalent to the average OD of the negative samples plus three times the standard deviation (SD) of the average OD and should be less than the limit of quantitation (LOQ).

3.4. Limit of quantitation (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of IgE (ng/ml) that can be quantified from the standard curve with a pre-defined precision. Usually, a variability of less than 20–25% of triplicate measurements, expressed as percentage of coefficient of variation (% CV), is acceptable. The '% CV' is calculated by the ratio of SD to the average OD and is expressed in per-

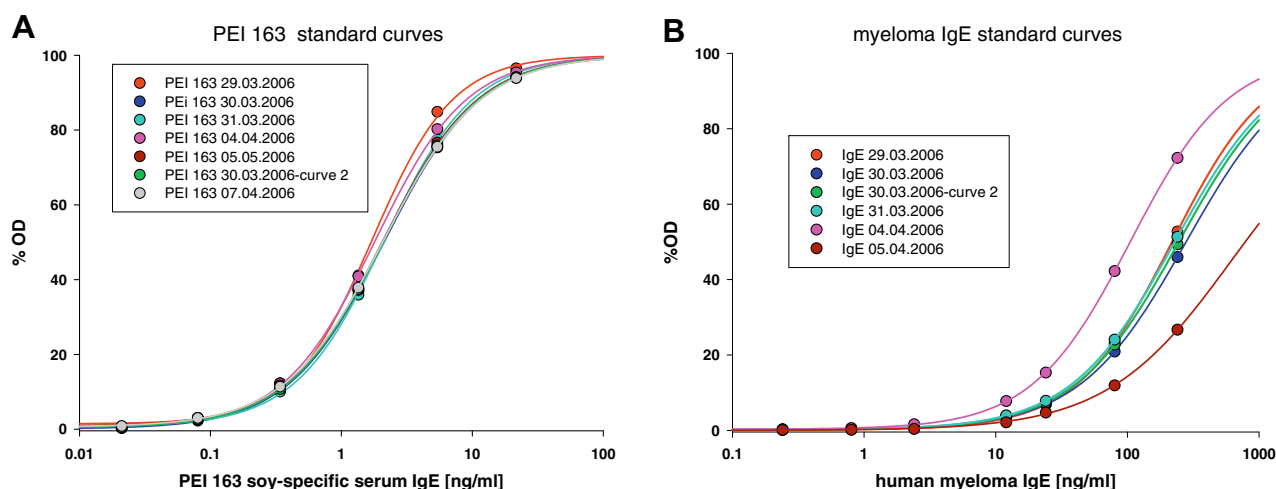


Fig. 1. Comparison of human serum (PEI 163) IgE standard curve (A) and human myeloma IgE standard curve (B). To better compare the standard curves created by each method, the ODs of the standards were expressed as percentage of the maximum OD of the individual standard curves.

centage. The LOQ must be greater than the LOD. Any values that fall between the LOQ and LOD can not be quantified because they do not fulfil the criterion of required precision.

4. Data analysis

Once the IgE ELISA has been validated with regard to sensitivity (LOD), limit of quantitation (LOQ), and precision, the confidence in the results of a specific serum screen will depend largely on the number of sera that are available for analysis. To achieve 95% certainty that a major allergen (a major allergen is defined as one to which more than 50% of individuals sensitive to that substance react in IgE-specific immunoassays) from the source material has not been transferred, a negative result must be obtained with at least 6 relevant sera. Furthermore, by using 17 relevant test sera, a 95% probability exists of detecting a minor allergen (a minor allergen is defined as one to which less than 50% of individuals sensitive to that substance react in IgE-specific immunoassays) from the source to which at least 20% of the affected population is reactive (FAO/WHO, 2001). An argument can be made for using fewer sera if relevant sera are not available, but this modified approach carries the risk of a false negative outcome. The use of larger numbers of less qualified sera has been advocated by some investigators. However, the use of these types of sera severely limits the probative value of the data generated from such studies. In cases where sera is limiting the use of a smaller number of very well documented, high quality sera is preferable to the use of larger numbers of lesser-quality sera.

The statistical model used to evaluate these types of studies should be based on a randomized complete block design where serum is the block and crop variety or protein represents the treatment. A test for non-additivity should be performed with Tukey's one degree of freedom test for non-additivity (Snedecor and Cochran, 1980) using an SAS macro developed by Oliver Schabenberger (SAS Institute). If the non-additivity test p -value < 0.0001 rejects the additivity assumption a randomized complete block design is not applicable to analyze the data and consequently an alternate analysis should be performed. This can occur when the population of allergic patients being tested have widely divergent amounts of allergen specific IgE, indicating that the population is not homogeneous. An alternate statistical analysis could be used that calculates a 99% tolerance interval with 95% confidence for individual sera using the IgE binding values obtained for reference extracts. The test substances IgE binding values can then be compared to the tolerance interval to determine where their IgE binding values fall in relation to the upper and lower tolerance values. Test substance IgE binding values that fall within the tolerance interval would not pose any additional allergenic risk to the affected population.

5. Additional considerations important to a successful IgE ELISA

To fully validate an IgE ELISA there are several additional points to consider that are unique to working with IgE. These include:

- Unspecific binding inherent to the IgE antibody – unspecific IgE binding to proteins and extracts can occur when inadequate blocking reagents have been used or when antibody to target ratios is inappropriate (Hoff et al., 2007).
- Blocking reagents – if blocking reagents such as non-fat dry milk or other bovine proteins are used to block unspecific binding it must be checked that IgE does not by itself react to the blocking reagent (Pedersen et al., 2004).

- Glycosylated proteins – low affinity binding to glycosylated proteins can give false positive results as described above (Altmann, 2007).
- Low concentration of IgE relative to IgG in patient sera – if the serum contains many IgG-antibodies to the protein in question, these may block for the detection of IgE binding.

6. Summary

In vitro IgE binding methods are an integral portion of the allergy safety assessment process and have proven to be invaluable when evaluating the safety of biotech products. To be maximally effective these methods should use clinically documented patient sera that have a specific allergy diagnosis. Use of less qualified sera can lead to false positive or false negative results that may have a detrimental effect on public health safety. In addition, test, control, and reference substances used in IgE binding studies should be adequately characterized to ensure their identity, purity, and concentration. Finally, the IgE binding method used to determine whether a biotech protein or biotech product poses an allergenic risk should be validated to ensure that they are precise, robust, and reproducible.

Conflict of interest statement

The author declares that there are no conflicts of interest. This work was funded by Monsanto Company.

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