Fraunhofer IZI

Fraunhofer Institute for Cell Therapy and Immunology IZI

Food allergen epitopes and peptide arrays

Allergy to tree nuts

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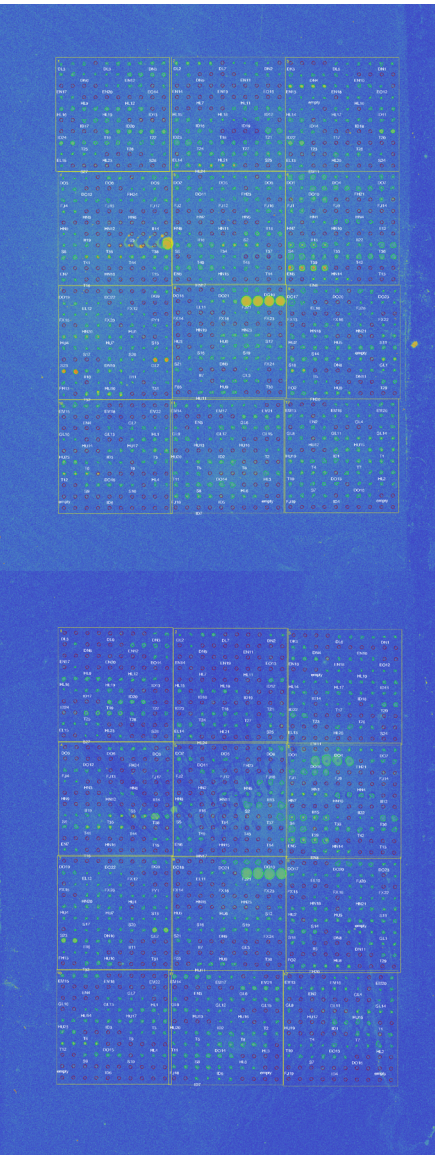
Food allergies

The prevalence of food allergies is increasing worldwide and therefore there is an urgent need of improving its diagnosis and the identification of clinically relevant biomarkers. Allergy to tree nuts alone, including hazelnut, walnut, cashew and almond, are accounted in almost 5 % of the global population. These allergies, together with peanut allergy, are responsible of more than 70 % of the reported food-induced fatalities.

Diagnosis and prognosis problem

Oral food challenge is the current gold standard for food allergy diagnosis. Although reliable, this method is challenging and even risky for patients with severe allergic disease. Though other biomarkers such basophil activation test or mast cell activation assays have shown diagnostic potential, their cost and complex implementation in the clinics still makes serum antibody-based biomarkers a convenient approach for food allergy diagnosis.

Although component-resolved diagnostics has improved the accuracy of allergen-specific sensitization measures, no correlation has been demonstrated between allergen-specific IgE concentrations and the severity of patients' food sensitization. Current molecular diagnostic approaches cannot distinguish between clinically and non-clinically relevant sensitization, especially regarding closely-related food. Cross-reactivity of allergens from



Array slide processed with a specially developed array imaging software: One food allergen patient serum with IgG (up) and IgE (down) measurement on an array with 320 peptides in fourfold. Peptides are taken from food allergies that are subject to declaration.

certain protein families, such PR-10, profilins or non-specific lipid transfer proteins leads to complex co-sensitization patterns. This occurs in patients with oral allergic syndrome, who are sensitized to multiple vegetables, fruits and pollen. Likewise, up to 30 % of the peanut allergic patients are allergic to at least another tree nut as well.

Epitope-based approaches may improve allergy serum diagnostics. Correlation between clinically relevant peanut and milk sensitization and patients' IgE epitope diversity has been reported. Epitope-based diagnostic and prognostic biomarkers could also improve personalized medicine approaches. In the future, allergen-specific immunotherapies may be improved by discriminating the patients who will benefit from the treatment from those who would not. It is therefore crucial to apply reliable epitope fingerprinting technologies together with a robust and costly-effective platform for measuring epitope recognition by patients' serum antibodies.

In Fraunhofer research projects hundreds of epitopes of food allergen antibodies from patient sera have been mapped and validated as short peptides.

Peptide arrays

Fingerprinting of potential epitopes in food allergen proteins including most of the food allergen that are necessary to declare has been carried out. This resulted in the successful identification of more than 300 potential epitopes. The array on the left shows a routine array for the characterization of patient sera with 320 peptides. The usage of epitopes determined at amino acid resolution results in patient-specific-epitope-based diagnosis and is therefore suitable to exclude possible cross-reactions.

Special glass slides have been developed that allow the coupling of peptides applying click chemistry. This allows to couple almost any peptide irrespective of its chemical characteristics and makes these arrays a versatile tool to explorer disease specific epitopes rapidly and with a large number of sera. In the ongoing studies more than 500 different sera have been analyzed for IgG and IgE binding.

Within a similar project a special array imaging software has been developed, which allows to identify and quantify the spot intensities on a single array in about 5 min instead of the approximate hour required by commercial semi-automatic software. Only with the help of this software the large number of samples investigated in this study could be handled.

The entire system allows the measurement of 160 patient samples within 36 hours. Slides are containing individual arrays from 16 x 90 spots (30 peptides) up to 2 x 1000 spots (300 peptides) and can be specifically manufactured for projects including our validated peptides epitopes. Depending on their design these are applicable to broad clinical studies as well as in depth allergy patient antibody screening.

Identification of peanut / hazelnut

Hazelnut and peanut allergies are two of the most threatening and frequent food allergens in Europe. As model to pre-validate the specificity of our peptide epitopes, we used sera from rabbits immunized with either hazelnut- or peanut-extracts. We observed that peptide epitopes recognized by immunized rabbit sera matched most epitopes recognized by sensitized patients' sera. IgG- and IgE-binding to peptide epitopes was tested using sera from patients reacting to food challenge with either hazelnut, peanut or both. These allergic patient cohort showed IgE- and IgG-binding to more peptide epitopes than sensitized patients. Patients allergic to peanuts or hazelnuts have different epitope recognition patterns than those allergic to both allergens. Our array contains allergen-specific peptide epitopes able to discriminate allergic / sensitized patients to either peanut or hazelnut. Currently, this set of peanut- and hazelnut-specific peptide epitopes are being used for the development of a point-of-care diagnostic device prototype.

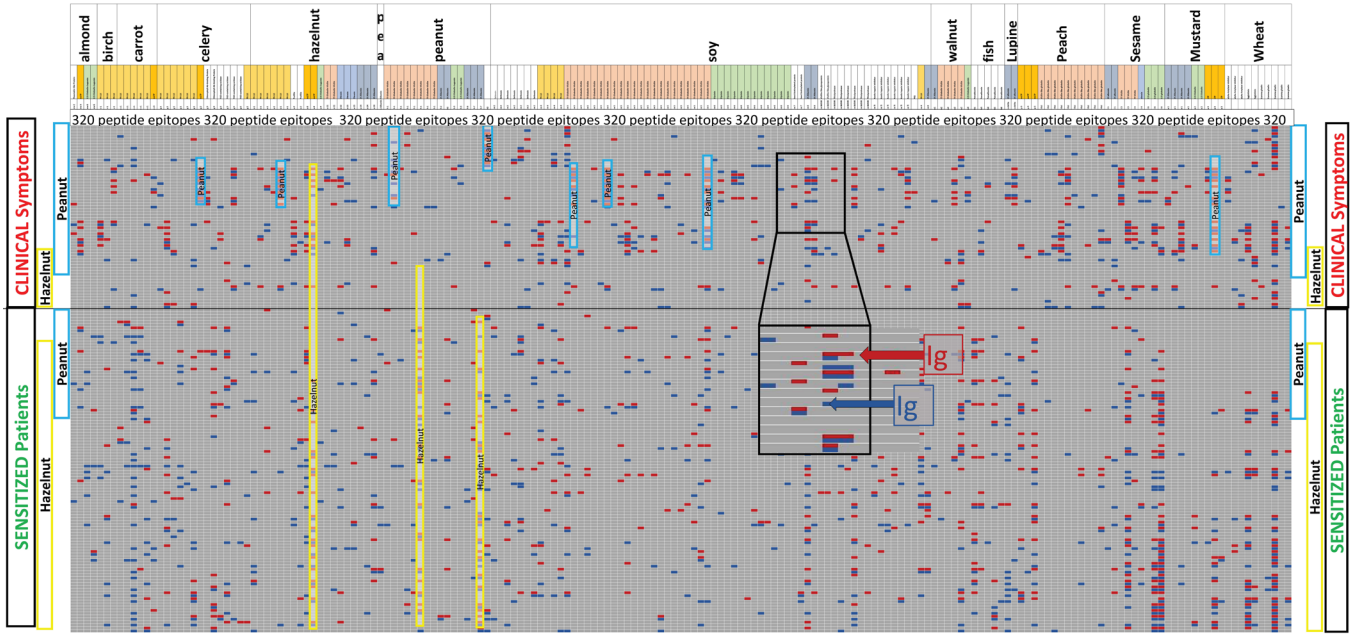
Differentiation of allergies (hazelnut walnut almond?)

Proteins of food allergens are phenotypically very variable, but their proteins are highly conserved. Due to their sequence similarity and conserved 3D structure, cross-reactions often occur and can have fatal consequences. Hazelnuts, for example, can trigger severe allergic symptoms. Hazelnut belongs to the tree nuts as well as walnut and almond. All tree nuts contain highly conserved proteins which can trigger cross reactions and allergic symptoms. Epitope-based diagnostics based on two to three peptides can discriminate between "tree nut allergy" patients and "true" hazelnut/walnut/almond allergy patients.

Predicting immunization outcome

Epitope-based diagnostics can potentially distinguish patients with clinically relevant sensitization. By using our present food-allergy peptide epitopes array, we reported that more than 80 % of soya sensitized patients with clinical reactions in their medical history could be distinguished from patients without symptoms (Kern et al., 2019). Beyond diagnosis, immunotherapies inducing long-term food de-sensitization are needed. Reported trials have shown encouraging, but still controversial results among patients of the same cohort. Prognostic serum biomarkers are therefore needed to distinguish patients who could benefit from allergen immunotherapy. Thus, we explored the diagnostic and / or prognostic potential of our peptide epitopes using sera from patients who participated in an allergen immunotherapy trial (EudraCT-Nr.: 2009-011737-27). Measurements of epitope binding patterns in patients' sera were analyzed in context of multiple clinical measures before and after treatment. We observed that patients with improved clinical measures at end of treatment recognized certain IgG peptide epitopes. Moreover, we showed the prognostic potential of the IgG recognition of two peptide epitopes in patients with decreased intensity of lip swelling after immunotherapy.

Analyses of food allergen array data: IgG (blue spots) and IgE (red spots) recognition pattern of allergic versus sensitized patients.



Publications

- Caballero LR et al. (2022). Peptide epitopes as bio-markers of soya sensitization in rBet v 1 immunotherapy of birch-related soya allergy. Clin Exp Allergy. Sep 14. Online ahead of print.
- Caballero LR et al. (2020). Identification of Seasonal Variations of Antibodies against PR-10-Specific Epitopes Can Be Improved Using Peptide-Phage Display. International Archives of Allergy and Immunology, 181(12), 919-925.
- Kern K et al. (2019). The immunome of soy bean allergy: Comprehensive identification and character-ization of epitopes. Clinical & Experimental Allergy, 49(2), 239-251.
- Havenith H et al. (2017). Combination of two epitope identification techniques enables the rational design of soy allergen Gly m 4 mutants. Biotechnology journal, 12(2), 1600441.

Method Background

Epitope fingerprinting

Epitope fingerprinting is a novel method developed at Fraunhofer IZI in collaboration with epitopic GmbH (Leipzig). Instead of generating novel peptide libraries or arrays specific for each antigen, which is the core of most comparable methods, epitope fingerprinting is applying an advanced combination of gene libraries, NGS and a specially developed software to map not only single antibodies but also the entire spectrum of antibodies found in a single drop of patient serum.

Advanced library design

A patented library design is the basis of a naïve peptide library on phage. This comprises more than 5 billion different 16mer sequences. The library has several unique advantages:

- Statistically well balanced, all sequences occur with the same frequency
- Covers almost all possible combinations of seven amino acids
- Allows constrained (Cys-flanked) as well as linear peptides in one library
- Stable in replication: Unlike similar biological libraries it can be replicated with minimal counterselection of "unfavorable" sequences

State of the art technologies

Peptides are selected by binding to antibody molecules. Because of the library's characteristics only one or two selection rounds are required. Working with peptide presenting phage only 50–100 need to be enriched, this increases the sensitivity. Only a few hundred antibody molecules are required to identify an epitope, when the selected peptide's genes are identified with NGS.

Novel software

A novel software was developed, which can handle the NGS data and helps to identify individual motifs:

- Reliable recognition of NGS sequencing and removal of suspicious sequences
- Statistical identification of peptide motifs, which is made possible by the reliable amino acid distribution in the patented peptide library
- Rapid comparison with potential antigens and a user-friendly JAVA interface that allows quick analyses of sequences containing potential epitope motifs
- Overall statistical analysis methods allow to identify unusual motifs for unknown targets or sequences enriched by binding to targets like cancer cells.

The final output of the typical analysis yields dozens and more enriched sequences sharing antigen motifs. These allow to assign the probably essential amino acids of an epitope. These are often surrounded by structurally relevant amino acids not present in the linear peptide of the antigen. Since the library peptides are 16 amino acids long and allow cysteines loop structures, unusual folds are often found directly from the analysis of the sequence alignment.

Validation of peptide epitopes in microarrays

Although the statistical predictions are usually conclusive for single antibodies or sera, the diagnostic value of a peptide epitope needs to be validated by analysis with multiple sera. For this purpose, a method was developed to immobilize any peptide irrespective of its individual chemistry on microarrays. This implies the use of click chemistry and allows to couple each peptide covalently through a linker to a glass slide surface in arrays of up to several hundred peptides. This is a routine method established by Fraunhofer IZI-BB in Potsdam. Minimal quantities of serum are required to measure binding of antibodies from the individual sera. The bound patient antibodies are detected with fluorescently labeled molecules and the arrays can by analyzed in any standard array reader.

A novel software for automatic analysis of array data has been created to overcome the bottleneck of semi manual evaluation. This allows to measure the epitope spectrum of hundreds of sera within a few days.

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