Published in final edited form as:

J Allergy Clin Immunol. 2009 August ; 124(2): 315–322.e3. doi:10.1016/j.jaci.2009.05.024.

Development of a Novel Peptide Microarray for Large Scale Epitope Mapping of Food Allergens

Jing Lin, PhD^a, Ludmilla Bardina, MSc^a, Wayne G. Shreffler, MD, PhD^a, Doerthe A. Andreae, MD^a, Yongchao Ge, PhD^b, Julie Wang, MD^a, Francesca M. Bruni, MD^c, Zhiyan Fu, PhD^a, Youngshin Han, PhD^d, and Hugh A. Sampson, MD^a

^aPediatric Allergy & Immunology, Mount Sinai School of Medicine, New York

^bDepartment of Neurology, Mount Sinai School of Medicine, New York

^cUniversity of Verona, Verona, Italy

^dSchool of Medicine, Sungkyunkwan University, Seoul, Korea

Abstract

Background—The peptide microarray is a novel assay which facilitates high-throughput screening of peptides with a small quantity of sample.

Objective—We sought to use overlapping peptides of milk allergenic proteins as a model system to establish a reliable and sensitive peptide microarray-based immunoassay for large scale epitope mapping of food allergens.

Methods—A milk peptide microarray was developed using commercially synthesized peptides (20-mers, 3 offset) covering the primary sequences of α_{s1} -, α_{s2} -, β -, and κ -caseins, and β -lactoglobulin. Conditions for printing and immunolabeling were optimized using a serum pool of five milk-allergic patients. Reproducibility of the milk peptide microarray was evaluated using replicate arrays immunolabeled with the serum pool, whereas specificity and sensitivity were assessed using serial dilution of the serum pool and a peptide inhibition assay.

Results—Our results show that epitopes identified by the peptide microarray were mostly consistent with those identified previously by SPOT membrane technology, but with specific binding to a few newly identified epitopes of milk allergens. Data from replicate arrays were reproducible ($R \geq 0.92$) regardless of printing lots, immunolabeling and serum pool batches. Using the serially diluted serum pool, we confirmed that IgE antibody binding detected in the array was specific. Peptide inhibition of IgE binding to the same peptide and overlapping peptides further confirmed the specificity of the array.

© 2009 Published by Mosby, Inc on behalf of American Academy of Allergy, Asthma and Immunology.

Corresponding author Jing Lin, PhD Pediatric Allergy & Immunology, Mount Sinai School of Medicine, Box 1198 New York, NY 10029-6574 Telephone: 212-241-3061 Fax: 212-426-1902 E-mail: jing.s.lin@mssm.edu.

Capsule summary: This is the first paper describing the optimization and validation of the peptide microarray-based immunoassay. It provides solid tools for current and future research for mapping of allergenic epitopes.

Clinical implications/Key messages:

- A reliable peptide microarray is established for large scale IgE epitope mapping.
- The peptide microarray-based immunoassay may provide an additional tool of food allergy diagnosis/prognosis.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conclusions—A reliable peptide microarray was established for large scale IgE epitope mapping of milk allergens and this robust technology could be applied for epitope mapping of other food allergens.

Keywords

Epitope mapping; peptide microarray; allergy; milk allergen

INTRODUCTION

Food allergy has emerged as an important public health problem in the United States.(1) Approximately 3.5% - 4% of Americans and 6-8% of children (<3 years old) are affected by IgE-mediated food allergy. Food allergy is currently diagnosed by clinical history and *in vivo* provocation tests (e.g. skin prick test, oral food challenges) or *in vitro* measurement of allergen-specific serum IgE (e.g. ELISA and CAP-System FEIA™ (Pharmacia Diagnostics AB, Uppsala, Sweden)).(2)

The double-blind, placebo-controlled oral food challenge (DBPCFC) is the “gold standard” for the diagnosis of food allergy.(1) However, it is time-consuming and there is a risk of life-threatening allergic reactions. An *in vitro* test to accurately predict the outcome of oral food challenges would be of great value. A number of studies have documented the correlation between IgE quantity and the likelihood of clinical allergy. Sampson and Ho(3), followed by several other groups(4-6) established 95% predictive decision points (food-specific IgE antibody levels measured by the CAP System FEIA) and suggested that an oral food challenge may not need to be performed in patients with suggestive histories of food allergy and specific IgE levels exceeding these values. However, the established levels vary widely between the patient populations studied, and there is a large ‘grey zone’ between high levels with good positive predictive values and undetectable levels that have good negative predictive values. Other variables such as the total IgE level and the ratio of allergen-specific IgE to total IgE have been considered for improved diagnostic precision, but also gave unsatisfactory results. (7) Another problem with current IgE testing is its limited application in the prognosis of food allergy. For example, the majority of pediatric patients with several forms of food allergy (e.g. milk, egg, wheat, soy), will outgrow their allergic conditions during childhood. However, current IgE testing cannot reliably predict whether patients will outgrow their food allergy.

Recent studies have suggested that clinical sensitivity, usually defined as a combination of reaction severity and the dose of allergen required to provoke a reaction to food allergens, may correlate better with epitope-specific recognition. For example, patients with persistent allergy or with a history of more severe allergic reactions to milk(8-10), peanut(11,12) and egg(13) were found to recognize a larger number of specific IgE sequential epitopes. IgE epitope mapping may become an additional tool for allergy diagnosis and prediction, and further contribute to the design of safe immunotherapeutics. Moreover, characterization of allergenic epitopes can lead to a better understanding of the pathogenesis and tolerance induction of food allergy.

In the past, epitope mapping was mainly performed using SPOT membrane-based immunoassays(10,14,15) in which the peptides are synthesized on the nitrocellulose membrane and then incubated with the patient’s sera. However, synthesis of large numbers of peptides is relatively error prone, time consuming, labor intensive and expensive, and has limitations due to specific chemistry of the method. A large volume of serum is required, and there is also a limitation for the number of targeted peptides. In addition, the SPOT technique is strictly qualitative, with very little statistical analysis possible due to the absence of replicates and small throughput.

With the development of microarray technology(16-18) and evolution in peptide synthesis techniques(19,20), we have been applying peptide microarray-based immunoassays for epitope mapping of several food allergens(12,21-23) because of its numerous advantages: It is possible to assay thousands of target peptides in parallel using small volumes of diluted serum, greatly reducing the biological sample cost of individual assays and allowing for more robust replication and statistical approaches for analysis and epitope determination. Several immunoglobulin subclasses other than IgE can be tested concurrently, allowing us to simultaneously investigate potential regulatory responses (e.g. IgG4, IgA and IgG1) that may influence clinical reactivity. However, a peptide microarray platform that measures specific binding and gives reproducible results has to be optimized and validated before it can be widely applied for epitope mapping of food allergens. In this paper, we describe the development and validation of a reliable peptide microarray-based immunoassay based on the peptides of milk allergens.

METHODS

Serum samples

A pool of the sera from 5 patients with diverse clinical symptoms, highly allergic to milk and with milk-specific IgE greater than 100 kU_A/L was prepared; the milk-specific IgE level of the pool was 850 kU_A/L as measured by UniCAP (Phadia, Uppsala, Sweden).

The serum of a non-atopic healthy donor was used as a negative control in all the experiments.

Peptide preparation

A library of peptides, consisting of 20 amino acids in length with an offset of 3 amino acids, corresponding to the primary sequences of α_{s1} -, α_{s2} -, β -, and κ -caseins, and β -lactoglobulin, was commercially synthesized (JPT Peptide Technologies GmbH, Berlin, Germany). The synthesized peptides were supplied as lyophilized powder with a purity of over 70% as analyzed by HPLC and Mass Spectrometry. Unless otherwise stated, peptides were resuspended in DMSO at 1 mg/mL, diluted 1:2 in Protein Printing Buffer (PPB, TeleChem International, Inc., Sunnyvale, CA) with 0.02% Sarkosyl to a final concentration of 0.5 mg/mL and stored in a 384 well assay plate, with low volume, non-binding, white polystyrene, round-bottom surface (Corning Incorporated, Corning, NY) covered with Robolid (Corning Incorporated) in a sealed plastic bag at -80°C until used.

Microarray printing

All peptide samples were printed on epoxy-derivatized glass slides (SuperEpoxy Substrate, TeleChem International, Inc.) using a NanoPrint™ Microarrayer 60 (TeleChem International, Inc.) equipped with 2×4 ArrayIt Stealth Micro Spotting Pin (SMP3B). All slides were used within six months of the manufacturing date and kept in a vacuum prior to printing.

Immunolabeling with Alexa based detection system

A rectangular incubation area around the printed grids was demarcated using a hydrophobic Dako Cytomation Pen (Glostrup, Denmark) and the following incubation steps were performed in a humidity chamber (Binding Site, Birmingham, UK) in the dark on a rotating platform (Labline) with gentle agitation. The slides were rinsed with PBST and non-specific binding sites blocked with 400 μ L of 1% HSA (96.99% purity, Sigma) in PBST (blocking buffer) for 1 hour at room temperature. After removing the blocking buffer from the slide surface by aspiration, 250 μ L of diluted serum pool or 1/5 diluted negative control in blocking buffer was applied and incubated on a rotator. A time course experiment in which arrays were incubated with either 1:50 or 1:500 diluted serum pool and the negative control serum for various time

points (1 h at room temperature, 4 h at room temperature, overnight and 24 h at 4°C) was performed to decide the optimal serum incubation conditions. In order to eliminate slide to slide variation, 3 arrays were run for each condition. Slides were then washed with PBST and incubated for 1 h at room temperature with polyclonal goat anti-human IgE diluted 1: 5000 (working concentration: 0.4 µg/mL, gift from Phadia), which was covalently conjugated with Alexa 546 (Molecular Probes — Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. It has fluorochrome:Ab molar ratios of 5-10 and has been validated by our group. Slides were washed with PBST, distilled water, and spin dried.

A peptide inhibition assay was carried out in which the array was immunolabeled as described above except that the serum pool (diluted 1:20 in blocking buffer) was pre-incubated with specific peptides for 1 h with agitation, and centrifuged before incubation with the array. Five different peptide inhibition groups containing 4-5 selected peptides in each group were tested (0.5 µg/250 µl for each peptide) with the serum pool on 5 replicate arrays. As the peptides were at 1 mg/mL in DMSO, the same serum pool pre-incubated with 2.5 µl of DMSO without peptides was applied onto a replicate array in parallel as control.

Data Analysis

The processed slides were scanned using a ScanArray®Gx (PerkinElmer, Waltham, MA) and images were saved as TIF files. Fluorescence signal was digitized with ScanArray Express (Perkin Elmer, Waltham, MA) and exported as comma-delimited text files. Data were analyzed using either a simple analysis in which a raw fluorescent value (expressed as digital fluorescence units [DFUs]) for each spot was calculated by subtracting local background from the median fluorescent value of the spot, or a more complex analysis (for immunolabeled slides) with R version 2.6.0 as follows: Briefly, the read-out (S_i) used for each spot, including the replicates for peptides and PPB, was the $\log_2$ transformation of the ratio of the median fluorescent signal of the spot to the median fluorescent signal of the local background. A “Z score” was calculated for each array element (spot) using PPB values within the same array (>400 spots/array):

$$Z_i = \frac{S_i - \text{Median}(S_{\text{PPB}})}{\text{MAD}(S_{\text{PPB}})}$$

By using the above equation, the read-out S_i for the array element was transformed into a Z score (Z_i). The $\text{Median}(S_{\text{PPB}})$ and $\text{MAD}(S_{\text{PPB}})$ were the Median and the Median Absolute Deviation (MAD) of all the read- outs of PPB spots, respectively. MAD is a robust version of the standard deviation. A detailed explanation of MAD can be found in the Online Repository.

An index Z value of each peptide element was generated from the Median of Z scores of the six replicate spots. An individual peptide sample was considered positive if its index exceeded 3, meaning that the signal is above the background with p value less than 0.3% (See Online Repository for detailed explanation). Further analysis and data presentation were also performed using Microsoft Excel, Sigmaplot and TIGR Multiexperiment Viewer (TMeV).

RESULTS

1. Optimization of printing conditions

1.1 Printing consistency—Since the ability of the printers to produce spots of the same samples with consistent fluorescent intensities is a key factor for the peptide microarray, a consistency experiment was carried-out (See Online Repository) to test the pin performance. As shown in Figure E1 in Online Repository, the raw fluorescent value was very consistent

from spot no.1 to 250 of pin no.1 to 8. In order to ensure printing quality, no more than 200 spots of samples were printed without reloading the pins in later experiments.

1.2 Effects of Humidity—The effect of humidity on printing was also tested (Figure E2 in Online Repository). Although the Alexa 546-BSA formed spots of similar sizes at different degrees of humidity, their fluorescent intensities significantly increased ($p<0.01$) as the humidity increased from 55% to 62%, suggesting that either more material was deposited on the slide or the epoxy had higher binding capacity at higher humidity. Therefore, all printing for subsequent experiments was performed at 62% humidity.

1.3 Printing buffer—When printed at 0.33 mg/ml in 33% DMSO in PPB, many of the milk peptides formed small spots with irregular shapes, which were much smaller than the spots of BSA-Alexa 546 (1.5 $\mu\text{g/ml}$ in PPB)(Figure E3A). The triplicate spots from each peptide were unevenly immobilized. Several solvents and detergents were tested in the printing buffer to improve spot morphology. The presence of 0.02% Sarkosyl increased the spot size (Figure E3B) and the peptide spots became more uniform when DMSO and peptide concentrations were increased together (Figure E3C). Therefore peptides were printed in 50% DMSO, 0.02% Sarkosyl in PPB in subsequent experiments.

2. Optimization of serum incubation conditions

Four conditions: 1 h and 4 h at room temperature, overnight (16 h) and 24 h at 4°C were tested for the optimal serum incubation times using 2 dilutions (1/50 and 1/500) of the serum pool. As shown in Figure 1, when the serum was diluted 1/500, 16 h incubation at 4°C seemed to be most sensitive, with ~33% of peptides showing positive reactions to the serum pool and only ~3% of peptides showing non-specific positive reactions with the negative control serum, while 1 h and 4 h incubation gave less than ~5% of peptides with positive reactions. Binding to peptides decreased and only ~21% peptides showed positive reactions when the incubation time was extended to 24 h under the same conditions (4°C). A similar maximal reaction (~79% positive peptides) was observed from the 1/50 diluted serum pool after 16 h incubation at 4°C, compared with ~30%, ~56%, and ~70% positive peptides after 1 h, 4 h, and 24 h incubation, respectively. Therefore, all subsequent incubations with sera were performed at 4°C overnight to increase sensitivity.

3. Reproducibility

The effect of three variables, printing lot, immunolabeling batch and serum pool batch, on reproducibility of the peptide microarray-based immunoassay was assessed using five replicate arrays (Array 1 to 5) immunolabeled with the serum pool. Among them, Array 1 and 2 were printed on the same day and immunolabeled on the same day with the IgE serum pool, which was freshly prepared from 5 patients' sera. From Array 3 to 5, each differed from Array 1 in one of the 3 variables: printing from the same printing plate on a separate day, immunolabeled on a separate day, or immunolabeled with the serum pool that was prepared 3 months earlier and went through several freeze-thaw cycles. The raw fluorescent values from the replicate arrays are presented in 4 scatter plots in Figure 2. Reproducibility was evaluated using Pearson's correlations ($p<0.01$).

As shown in Figure 2, the raw fluorescent values from the arrays with the same printing lot, immunolabeling day and serum pool batch were very reproducible, with very high correlation coefficients ($R=0.96$). Immunolabeling day and serum pool batch did not affect the reproducibility; and both had correlation coefficients of 0.95. Although the printing lot appeared to have a comparably greater effect on reproducibility, it still had a correlation coefficient of 0.92.

4. Specificity and sensitivity of the peptide microarray-based immunoassay

Specificity and sensitivity of the peptide microarray-based immunoassay were assessed using a serial dilution of the serum pool, and specificity was further confirmed by peptide inhibition assays.

4.1 Serum dilution experiment—Six replicate slides were immunolabeled with the negative control serum (diluted 1:5) and the serum pool at dilutions of 1:5, 1:10, 1:100, 1:1000, and 1:10000. Data were analyzed using R and presented using TMeV (Figure 3A). When diluted 1/5, the serum pool showed strong binding to the peptides. Most of the positive binding observed was not to a single isolated peptide, but to several overlapping peptides, suggesting the presence of an allergenic epitope. Since the peptides overlapped by 17 amino acids, it is possible to identify IgE epitopes based on the overlapping amino acid sequences. Several major epitopes in these 5 milk allergens have been previously identified using SPOT membrane immunoassays and a detailed comparison between the results from the peptide microarray and SPOT membrane immunoassays is shown in Figure 4.

As shown in Figure 3A, the IgE binding to various peptides using the 1:5 diluted serum pool decreased, and in some cases disappeared when the pool was further diluted. Most of the epitopes remained detectable at a 1/100 dilution, but many of them could not be detected at a dilution of 1/1000. Almost no peptide showed positive binding with the serum pool diluted to 1/10000 or with negative control serum, indicating that most of the signal to peptides utilizing serum at 1/5 dilution is specific. There are a few peptides of α_{s1} - and α_{s2} -casein which remained positive or showed greater positive signal when the pool was diluted, suggesting non-specific binding to these peptides. In total, there were less than 10 peptides out of 289 milk peptides that showed non-specific signal.

4.2 Peptide inhibition experiment—In order to test the specificity of the IgE binding, a peptide inhibition assay was performed. Preliminary experiments performed in our lab have shown that peptides, either in a mixture with other peptides or as a single peptide, exhibited similar inhibition effects on IgE binding to the same peptide and neighboring peptides. Therefore 5 different peptide inhibition groups, each containing 4-5 separate peptides found to bind patient IgE in the serum dilution experiment, were selected and tested on 5 replicate arrays.

As shown in Figure 3B, compared with the IgE reactions from the control array, which was incubated with the serum pool without any inhibiting peptide, all the peptides in the 5-peptide inhibition groups inhibited 100% of the IgE binding to the same peptide (targeted peptide). In addition, IgE binding to at least one neighboring peptide was also inhibited. Interestingly, signal reduction from peptides other than the targeted peptides was observed, indicating either non-specific inhibition or some similarity between the peptide sequences (Figure 3B).

DISCUSSION

Microarray technology has been widely used for studying gene expression and regulation for almost two decades.(24) Because of its high throughput, low cost, low sample requirement, and capacity for simultaneous testing of many different targets, microarray has been applied in allergy studies and a miniaturized allergy test in microarray format was developed in 2002. (16) for quantitative measurement of serum allergen-specific IgE levels. It has been shown in later studies that results obtained from these microarrays are consistent with other established methods, such as RAST, ELISA and immunoblot tests.(25,26) The microarray system was adapted for studying sequential allergenic epitopes in our laboratory by Shreffler et al.(12) The importance of epitope recognition in allergic patients has been previously demonstrated. (9, 12,13) Using a microarray format, it is possible to use overlapping peptides representing

putative epitopes, which greatly increases the accuracy of the test. This technology is proving to be attractive for a range of studies, and we have employed peptide microarrays in the mapping of IgE and/or IgG4 epitopes of allergens in peanut, milk and egg with encouraging results. However, the peptide microarray system needs to be optimized and validated before it can be extensively used for allergy testing and various applications.

A typical peptide microarray consists of two experimental steps: printing of the peptides onto epoxide substrate (glass slide) in which the peptides bind covalently to the epoxide groups on the slide surface; and immunolabeling of the slides with patients' sera. We therefore attempted to optimize the system by improving the conditions for these two steps. Printing conditions including pin printing performance, effect of humidity, and printing buffer for peptides were first tested and optimized. With the adjusted conditions, we are able to print identical peptide spots with consistent signals. For optimization of immunolabeling, we have tested four conditions, varying from 1 h to 24 h at either room temperature or 4°C. As the milk specific IgE level varies between individuals, we used two different dilutions of the serum pool when investigating the serum incubation time. The greatest binding capacity was observed with overnight incubation at 4°C for both 1/50 and 1/500 dilutions, indicating that the binding capacity was not affected by the milk-specific IgE level.

The ability to obtain reproducible results is a prerequisite for any established test; we therefore determined reproducibility on replicate arrays, and the effect of different printing lots and immunolabeling batches. A study conducted in our laboratory using CAP System FEIA showed that multiple freeze-thaw cycles had little impact on IgE values for clinical decision making and a modest effect for research studies (Mishoe, M et. al, unpublished). Here our results confirmed that the peptide-specific IgE antibodies were not affected by multiple freeze-thaw cycles of the serum and that data collected from the microarray assay were very reproducible ($R \geq 0.92$) with similar reaction levels.

Another important aspect of the peptide microarray assay is specificity, which was confirmed in the serum dilution experiment and peptide inhibition assay. In the peptide inhibition assay, some non-specific inhibition was observed. Investigation of peptides from different inhibition groups showed that several inhibitory reactions were likely due to cross-inhibition by peptides with >35% sequence identity (Table E1). Although peptides with lesser similarity, i.e. in the twilight zone (15-35% sequence identity), were not considered here, they may be still cross-inhibiting certain peptides because structural patterns are frequently more recognizable than sequential patterns.(27)

Epitopes in milk allergens have been previously identified using SPOT membrane immunoassays.(15,28-31) Although the sera used in our study are different from those used previously with SPOT membrane immunoassays, results from these two systems seemed quite consistent. Most of the previously reported epitopes were recognized by our microarray system. In addition, we observed specific positive reactions from regions with no overlap or less than eight amino acids(32) overlapping with previously reported epitopes. Based on the overlapped amino acids, several new epitopes were identified (α_{s1} -casein: AAs 49-62; α_{s2} -casein: AAs 69-77; β -casein: AAs 16-39 and β -lactoglobulin: AAs 106-119)

There are several reasons that peptide microarray may be more accurate and sensitive than SPOT membrane immunoassays. In microarray technology, 10 fmol of peptide is bound to one mm^2 of the epoxide surface, whereas in SPOT membranes, it is a hundred times more — 1 nmol for the same area.(33,34) However, in the peptide microarray, the purity of the peptides bound to the slide can be strictly controlled with HPLC purification and Mass spectrometry analysis. At the same time, with SPOT membranes, there are many by-products due to the limitations of this *in situ* peptide synthesis technique. For example, hydrophobic amino acids

may not bind to the membrane and therefore are absent in the peptide sequence. The more hydrophobic amino acids in the sequence, the lower the purity. Therefore, there may be both an excess of non-specific binding and lack of specific binding, which greatly affects the signal to noise ratio.

In conclusion, we have established a reliable peptide microarray for large scale IgE epitope mapping of milk allergens. This robust technology can be applied for epitope mapping of other food allergens and provide information that could provide both diagnostic and prognostic value in food allergy testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

We would like to thank Todd Martinsky, Telechem International Inc. for valuable advice and continuous technical support; Rong Wang, Mount Sinai School of Medicine, for insights in peptide chemistry; and Shoshana Biro, CliniWorks for data analysis.

Declaration of all sources of funding: Y Han was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-214-C00099). H Sampson and J Lin were supported in part by a grant from NIAID (AI-44236). Studies were supported in part by a grant from the NIAID [AI-44236] and from the National Center for Research Resources (MO1-RR-00071).

Abbreviations used

DBPCFC, Double-Blind, Placebo-Controlled Oral Food Challenge; PPB, Protein Printing Buffer; MAD, Median Absolute Deviation; TMeV, TIGR Multiexperiment Viewer.

References

1. Sampson HA. Food allergy - accurately identifying clinical reactivity. *Allergy* 2005;60:19–24. [PubMed: 15842229]
2. Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* May;2001 107(5):891–6. [PubMed: 11344358]
3. Sampson HA, Ho DG. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J Allergy Clin Immunol* 1997;100(4):444–51. [PubMed: 9338535]
4. Garcia-Ara C, Boyano-Martinez T, Diaz-Pena JM, Martin-Munoz F, Reche-Frutos M, Martin-Esteban M. Specific IgE levels in the diagnosis of immediate hypersensitivity to cows' milk protein in the infant. *J Allergy Clin Immunol* 2001;107(1):185–90. [PubMed: 11150010]
5. Martinez, T Boyano; Garcia-Ara, C.; Diaz-Pena, JM.; Munoz, FM.; Sanchez, G Garcia; Esteban, MM. Validity of specific IgE antibodies in children with egg allergy. *Clin Exp Allergy* 2001;31(9):1464–9. [PubMed: 11591198]
6. Celik-Bilgili S, Mehl A, Verstege A, Staden U, Nocon M, Beyer K, et al. The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. *Clin Exp Allergy* 2005;35(3):268–73. [PubMed: 15784102]
7. Mehl A, Verstege A, Staden U, Kulig M, Nocon M, Beyer K, et al. Utility of the ratio of food-specific IgE/total IgE in predicting symptomatic food allergy in children. *Allergy* 2005;60(8):1034–9. [PubMed: 15969684]
8. Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* Aug;2002 110(2):293–7. [PubMed: 12170271]

9. Beyer K, Jarvinen KM, Bardina L, Mishoe M, Turjanmaa K, Niggemann B, et al. IgE-binding peptides coupled to a commercial matrix as a diagnostic instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2005;116(3):704–5. [PubMed: 16159646]
10. Chatchatee P, Jarvinen KM, Bardina L, Beyer K, Sampson HA. Identification of IgE- and IgG-binding epitopes on alpha(s1)-casein: Differences in patients with persistent and transient cow's milk allergy. *J Allergy Clin Immunol* 2001;107(2):379–83. [PubMed: 11174208]
11. Beyer K, Ellman-Grunther L, Jarvinen KM, Wood RA, Hourihane J, Sampson HA. Measurement of peptide-specific IgE as an additional tool in identifying patients with clinical reactivity to peanuts. *J Allergy Clin Immunol* 2003;112(1):202–7. [PubMed: 12847500]
12. Shreffler WG, Beyer K, Chu THT, Burks AW, Sampson HA. Microarray immunoassay: Association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* Apr;2004 113(4):776–82. [PubMed: 15100687]
13. Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA. Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy* 2007;62(7):758–65. [PubMed: 17573723]
14. Frank R. The SPOT synthesis technique - Synthetic peptide arrays on membrane supports - principles and applications. *J Immunol Methods* Sep;2002 267(1):13–26. [PubMed: 12135797]
15. Chatchatee P, Jarvinen KM, Bardina L, Vila L, Beyer K, Sampson HA. Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin Exp Allergy* Aug; 2001 31(8):1256–62. [PubMed: 11529896]
16. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, et al. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *Faseb Journal* Jan;2002 16(1):U262–U82.
17. Harwanegg C, Laffer S, Hiller R, Mueller MW, Kraft D, Spitzauer S, et al. Microarrayed recombinant allergens for diagnosis of allergy. *Clin Exp Allergy* Jan;2003 33(1):7–13. [PubMed: 12534543]
18. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science* Sep 8;2000 289(5485):1760–3. [PubMed: 10976071]
19. Panicker RC, Huang X, Yao SQ. Recent advances in peptide-based microarray technologies. *Combinatorial Chemistry & High Throughput Screening* Sep;2004 7(6):547–56. [PubMed: 15379626]
20. Shin DS, Kim DH, Chung WJ, Lee YS. Combinatorial solid phase peptide synthesis and bioassays. *Journal of Biochemistry and Molecular Biology* Sep 30;2005 38(5):517–25. [PubMed: 16202229]
21. Shreffler WG, Lencer DA, Bardina L, Sampson HA. IgE and IgG(4) epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. *J Allergy Clin Immunol* Oct;2005 116(4):893–9. [PubMed: 16210066]
22. Flinterman AE, Knol EF, Lencer DA, Bardina L, Jager C, Lin J, et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. *J Allergy Clin Immunol* 2008;121(3):737–43. [PubMed: 18234310]
23. Cerecedo I, Zamora J, Shreffler WG, Lin J, Bardina L, Dieguez MC, et al. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J Allergy Clin Immunol* 2008;122(3):589–94. [PubMed: 18774394]
24. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. *Science* 1995;270(5235):467–70. [PubMed: 7569999]
25. Lin J, Shewry PR, Archer DB, Beyer K, Niggemann B, Haas H, et al. The Potential Allergenicity of Two 2S Albumins from Soybean (Glycine max): A Protein Microarray Approach. *Int Arch Allergy Immunol* Jul 11;2006 141(2):91–102. [PubMed: 16837790]
26. Kim TE, Park SW, Cho NY, Choi SY, Yong TS, Nahm BH, et al. Quantitative measurement of serum allergen-specific IgE on protein chip. *Experimental and Molecular Medicine* May 31;2002 34(2): 152–8. [PubMed: 12085991]
27. Fu, Z. *Post-Structural Genomics: Correlate Protein Sequence, Structure, and Function*. VDM Verlag Dr. Müller; 2008.
28. Busse PJ, Jarvinen KM, Vila L, Beyer K, Sampson HA. Identification of sequential IgE-binding epitopes on bovine alpha(s2)-casein in cow's milk allergic patients. *Int Arch Allergy Immunol* 2002;129(1):93–6. [PubMed: 12373003]

29. Han N, Jarvinen KM, Cocco RR, Busse PJ, Sampson HA, Beyer K. Identification of amino acids critical for IgE-binding to sequential epitopes of bovine kappa-casein and the similarity of these epitopes to the corresponding human kappa-casein sequence. *Allergy* 2008;63(2):198–204. [PubMed: 18186809]
30. Jarvinen KM, Chatchatee P, Bardina L, Beyer K, Sampson HA. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int Arch Allergy Immunol* 2001;126(2):111–8. [PubMed: 11729348]
31. Cocco RR, Jarvinen KM, Sampson HA, Beyer K. Mutational analysis of major, sequential IgE-binding epitopes in alpha(s1)-casein, a major cow's milk allergen. *J Allergy Clin Immunol* 2003;112(2):433–7. [PubMed: 12897753]
32. Bischoff, SC.; Sellge, G. Immune Mechanisms in Food-Induced Disease. In: Metcalfe, DD.; Sampson, HA.; Simon, RA., editors. *Food Allergy: Adverse Reactions to Foods and Food Additives*. Blackwell Publishing; MA: 2003.
33. Wenschuh H, Volkmer-Engert R, Schmidt M, Schulz M, Schneider-Mergener J, Reineke U. Coherent membrane supports for parallel microsynthesis and screening of bioactive peptides. *Biopolymers* 2000;55(3):188–206. [PubMed: 11074414]
34. Reineke, U.; J., S-M.; Schutkowski, M. Peptide Arrays, Review article in BioMEMS and Biomedical Nanotechnology. In: Ozkan, M.; Heller, MJ., editors. *Micro and Nano-Technologies for Genomics and Proteomics*. Springer Science+Business Media, LLC; New York: 2006. p. 161-282.

Figure 1.

Time course experiment for optimization of serum incubation. 3 replicate arrays were immunolabeled with 1/500 diluted serum pool or 1/5 diluted negative control serum for each condition. Data presented are averaged Z scores of the replicates. Reactions to the pool and negative control serum are showed as $\blacklozenge$ and $\square$, respectively. The percentage of positive peptides (Z score > 3) are indicated.

Figure 2.

Scatter plots showing the effects of different variables (printing lot, immunolabeling day and serum pool batch) on reproducibility of the peptide microarray. A, data from two replicate arrays with the same 3 variables; B-D, data from two replicate arrays differing in one of the 3 variables: immunolabeling day (B), serum pool batch (C) or printing lot (D). Coefficient of correlation was calculated ($p < 0.01$) and shown in each scatter plot.

Figure 3.

Results from serum dilution experiment (A) and peptide inhibition assay (B). Data are presented as Z scores using TMeV. Areas showing non-specific binding are indicated with black arrows. Targeted peptides, which are the peptides pre-incubated with the serum pool are indicated with red arrows for each inhibition group. Areas with possible cross-inhibition based on sequence alignment are indicated with blue arrows.

Figure 4.

Comparison between the epitopes identified from SPOT membrane immunoassays and peptide microarray. Peptides with amino acid sequences of at least 90% overlapped with the major epitopes and minor epitopes identified using SPOT membrane technology are shown in and , respectively, while peptides with positive reactions (z score >3) to positive serum pool in peptide microarray are shown in .