Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray–based immunoassay

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Background: Peptide microarray analysis is a novel method that can provide useful information on the nature of specific allergies.

Objective: We sought to determine the specificity and diversity of IgE and IgG4 antibodies binding to sequential epitopes of α_{s1}-, α_{s2}-, β-, and κ-caseins and β-lactoglobulin by using a peptide microarray–based immunoassay.

Methods: A microarray immunoassay was performed with sera from 31 children with IgE-mediated milk allergy (16 with positive oral milk challenge results [ie, the reactive group] and 15 with negative oral milk challenge results [ie, the tolerant group]). A library of peptides, consisting of 20 amino acids (AAs) overlapping by 17 (3-offset), corresponding to the primary sequences of α_{s1}-, α_{s2}-, β-, and κ-caseins and β-lactoglobulin was printed on epoxy-coated slides. A region was defined as an epitope if it was statistically associated with reactive groups and recognized by at least 75% of reactive patients.

Results: By using this method, a total of 10 epitopes were identified: α_{s1}, AAs 28 to 50, 75% reactive and 26.7% tolerant; α_{s2}, AAs 1 to 20, 75% reactive and 13.3% tolerant; AAs 13 to 32, 75% reactive and 26.7% tolerant; AAs 67 to 86, 75% reactive and 33.3% tolerant; and AAs 181 to 207, 75% reactive and 20% tolerant; β-*casein*, AAs 25 to 50, 75% reactive and 33.3% tolerant, AAs 52 to 74, 81.3% reactive and 26.7% tolerant; and AAs 154 to 173, 75% reactive and 33.3% tolerant; β-*lactoglobulin*, AAs 58 to 77, 81.3% reactive and 40% tolerant; and κ-*casein*, AAs 34 to 53, 87.5% reactive and 40% tolerant.

Conclusion: Several regions have been defined as epitopes, which showed differential recognition patterns between reactive and tolerant patients. Further studies are needed to validate the utility of this assay in clinical practice. (J Allergy Clin Immunol 2008;122:589-94.)

Key words: *Milk allergy, peptide microarray, cow's milk proteins, oral tolerance, natural history, children, casein, α-lactalbumin, β-lactoglobulin*

Cow's milk allergy is a common and often transitory disease affecting approximately 2.5% of children less than 2 years of age.[1,2] The mainstay of treating milk allergy consists of prescribing a milk-free diet and having the patient return for regular follow-up visits to re-evaluate the status of his or her allergy. The majority of children with IgE-mediated milk allergy become tolerant. Depending on the series, rates of development of tolerance have varied: 76% by age 3 years,[2] 74% by age 5 years,[3] 22% by age 18 months to 13 years,[4] and 19% by age 4 years.[5] Additionally, 35% have allergic reactions to other foods, and approximately 50% have respiratory allergy.[2,6-8] At the outset, the difficulty lies in predicting which children might have severe allergic reactions to milk and which will remain allergic for a lifetime.[9,10]

Milk proteins are among the best characterized food allergens. The casein fraction is comprised of 4 different proteins: α_{s1}-, α_{s2}-, β-, and κ-casein in proportions of 40:10:40:10, respectively. The primary structures are known for most genetic variants of α_{s1}-, α_{s2}-, β- and κ-casein; β-lactoglobulin; and α-lactoalbumin.[11-14]

Previous work has increased our understanding of the importance of epitope recognition in the persistence of cow's milk allergy.[15-27] Food allergy is mediated by specific IgE antibodies directed toward specific food allergenic epitopes. IgE binding to both sequential and conformational epitopes can trigger allergic reactions. Patients with IgE antibodies that recognize sequential epitopes, as demonstrated by binding to greater numbers of synthetic peptides from relevant allergen sequences, react to foods in any form (ie, even when extensively cooked) and tend to have more persistent allergy.[15-27] Most of the data regarding IgE epitopes of food proteins to date have been generated through the use of SPOTS membrane-based immunoassays (Genosys Biotechnologies, Woodlands, Tex).[28] However, for the characterization of large numbers of individual-patient allergen-specific IgE antibodies, this method has technical disadvantages. The synthesis of large numbers of peptides is relatively time-consuming, labor intensive, and expensive; assays require milliliter quantities of patient sera; and the membranes can be used for a limited number of patients.

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Abbreviations used
AA: Amino acid
HSA: Human serum albumin
PBS-T: PBS–0.5% tween 20
SNR: Signal/noise ratio
SPT: Skin prick test

Shreffler et al[29] have developed a peptide microarray immunoassay to examine serum samples from patients with peanut allergy and confirmed that allergenic/antigenic areas identified by using this method correlated with areas defined by SPOTS membrane mapping. Furthermore, the diversity of epitope recognition was found to correlate with the severity of peanut-induced allergic reactions.[30] More interestingly, an additional epitope, not previously identified as important for peanut allergy, was discovered by using this method. The success of using microarray assays in elucidating the importance of individual peanut epitopes in peanut allergy prompted us to expand our search for similarly important milk protein epitopes. The aim of this study was to determine the IgG and IgE specificity and diversity to sequential epitopes of α_{s1}-, α_{s2}-, β-, and κ-caseins and β-lactoglobulin by using a peptide microarray–based immunoassay in a series of pediatric patients with cow's milk allergy.

METHODS

Patients

Patients with cow's milk allergy were recruited from the Hospital Ramon y Cajal Allergy Clinic from January 2005 until February 2006. The diagnosis of IgE-mediated milk allergy was made on the basis of the clinical history and positive skin prick test (SPT) responses with milk and α-lactalbumin, β-lactoglobulin, and casein and/or specific IgE to milk and its protein fractions measured by using the CAP System FEIA (Phadia, Uppsala, Sweden). Oral milk challenges were performed if the history was unclear.[31,32] Patients given diagnoses were started on a milk exclusion diet for 6 months. After the milk-free diet, SPT responses and specific IgE levels were re-evaluated. Those patients with IgE levels to milk of less than 2.5 kUA/L, according to literature recommendations,[33,34] were scheduled for an oral milk challenge. A challenge result was considered positive if cutaneous symptoms (urticaria and angioedema), digestive symptoms, and/or respiratory symptoms were observed within 2 hours after any dose of milk. Children experiencing unequivocal allergic symptoms after an accidental ingestion of milk at home were also considered as having a positive challenge result and were included in the reactive group. Patients considered nonreactive had tolerance confirmed with a milk challenge at the hospital.

Peptides

A library of peptides, consisting of 20 amino acids (AAs) overlapping by 17 (3-offset) and corresponding to the primary sequences of α_{s1}-, α_{s2}, β-, and κ-caseins and β-lactoglobulin, was commercially synthesized (JPT Peptide Technologies, Berlin, Germany). Peptides were resuspended at a concentration of 150 μmol/L in Protein Printing Buffer (ArrayIt, Sunnyvale, Calif) with 33% dimethyl sulfoxide and printed in triplicate to epoxy-derivatized glass slides (SuperEpoxy Substrate, ArrayIt) by using the NanoPrint Microarrayer 60 (TeleChem International, Inc, Sunnyvale, Calif). Slides were stored at 4°C until use. Additional array elements, including purified α_{s1}-, α_{s2}-, β-, and κ-caseins and β-lactoglobulin, were used as positive controls, Protein Printing Buffer alone was used as a negative control and for background normalization, and fluorochrome-labeled BSA for the purpose of grid alignment during analysis was included on each slide. All array elements were printed in duplicate (2 sets of triplicates) to improve precision and to determine intra-assay variation.

Immunoassay

Immunolabeling was performed as previously described, with minor modifications.[29,35] In brief, an area around the arrays was demarcated with a hydrophobic pen (DakoCytomation Pen; Dako, Glostrup, Denmark). The slides were then rinsed with PBS–0.5% tween 20 (PBS-T) and nonspecific binding sites blocked with 500 μL of 1% human serum albumin (HSA) in PBS-T (PBS-T/HSA) for 60 minutes at room temperature on a rotating platform (Labline). Incubations were performed in a humidity chamber (The Binding Site, San Diego, Calif). After removing the PBS-T/HSA from the slide surface by means of aspiration, 200 μL of patient sera diluted 1:5 in PBS-T/HSA was applied and allowed to incubate for 60 minutes on a rotator at room temperature. Slides were then washed with PBS-T and incubated for 1 hour at room temperature with a 1:1 mixture of monoclonal mouse anti-human IgE (Phadia) and IgG4 (PharMingen, San Diego, Calif) diluted in PBS-T/HSA (working concentration, 0.4 μg/mL), which had been covalently tagged with fluorochromes Alexa 546 and Alexa 647 (Molecular Probes, Eugene, Ore), respectively. Slides were then washed with PBS-T, centrifuged dry, and scanned with a ScanArrayGx (PerkinElmer, Fremont, Calif). Images were saved as TIFF files.

Data analysis

Fluorescence signals from 2 different channels (for IgE and IgG4) were digitized with the ScanArray Express Microarray Analysis System (PerkinElmer), exported as ASCII comma-delimited files. The intensity for each spot was expressed as a signal/noise ratio (SNR). R software (version 2.5.0. R Project Foundation) was used to normalize the data. Individual array element values with higher coefficients of variation were eliminated from analysis. Intraslide negative controls were used to define minimum background binding intensity for positive elements. For intra-assay comparison, values were log10 transformed for normalization and homogenization of variance and then adjusted to set the median from each slide equal to 1. In subsequent analyses only ratios of IgE to IgG4 SNR intensities greater than a minimum threshold of 1 were considered positive. In this study we enrolled only subjects with low milk-specific IgE levels and similar clinical features; the only difference between the groups was the outcome of the oral milk challenge. Consequently, we focused on IgE-binding regions that correlated with the challenge outcome. Understandably, there is a risk of missing some allergenic regions by using this approach, but we wished to focus on specificity. It has been shown that patients with food allergy who become tolerant to a specific food have higher food-specific IgG4/IgE ratios compared with those with persisting reactivity. Furthermore, it has been postulated that the maintenance of tolerance to cow's milk in atopic children could be associated with increased levels of specific IgG4.[36,37] By evaluating IgE/IgG4, we sought to focus on clinically relevant epitopes because we wished to differentiate between tolerant and reactive patients.

Absolute and relative frequencies of patients' antibodies recognizing each epitope were determined. Peptides that were bound by antibodies from greater than 75% of patients with milk allergy were considered epitopes. Among them, we further looked for those epitopes statistically associated with clinical reactivity. The Fisher exact test was used to determine statistical associations, and a P value of .05 or less was considered significant.

RESULTS

Thirty-one patients fulfilling the above criteria were identified in the allergy clinic between January 2005 and February 2006 and were included in the study. The median age was 2 years and 4 months (range, 1-6 years). The most frequent presentation at diagnosis was isolated cutaneous symptoms (urticaria) in 58.6% of the patients, followed by urticaria and vomiting in 25.8%. Oral