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Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes

Emma M. Savilahti, MD^a, Ville Rantanen, MSc^b, Jing S. Lin, PhD^c, Sirkku Karinen, MSc^b, Kristiina M. Saarinen, MD PhD^a, Marina Goldis, MD^c, Mika J. Mäkelä, MD PhD^d, Sampsa Hautaniemi, DTech^b, Erkki Savilahti, MD PhD^a, and Hugh Sampson, MD^c

^a Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland ^b Computational Systems Biology Laboratory, Institute of Biomedicine and Genome-Scale Biology Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland ^c The Division of Pediatric Allergy and the Jaffe Institute for Food Allergy, Mount Sinai School of Medicine, New York ^d Hospital for Allergic and Skin Diseases, University of Helsinki, Helsinki, Finland

Abstract

Background—Dynamics and balance of allergen specific IgE, IgG4 and IgA binding may contribute to the development of tolerance in cow's milk allergy. Profiling of antibody binding to cow's milk protein epitopes may help in predicting natural history of allergy.

Objective—To investigate differences in IgE, IgG4 and IgA binding to cow's milk epitopes over time between patients with early recovery or with persisting cow's milk allergy.

Methods—We studied serum samples at the time of diagnosis (mean age 7 months), one year later and at follow-up (mean age 8.6 years) from 11 patients with persisting IgE-mediated cow's milk allergy at age 8-9 years, and 12 patients who recovered by age 3 years. We measured the binding of IgE, IgG4 and IgA antibodies to sequential epitopes derived from five major cow's milk proteins with a peptide microarray-based immunoassay. We analyzed the data with a novel image processing method together with machine learning prediction.

Results—IgE epitope binding patterns were stable over time in patients with persisting cow's milk allergy, whereas binding decreased in patients who recovered early. Binding patterns of IgE and IgG4 overlapped. Among patients who recovered early, the signal of IgG4 binding increased while that of IgE decreased over time. IgE and IgG4 binding to a panel of α s1-, α s2-, β - and κ -casein regions predicted outcome with significant accuracy.

Conclusions—Attaining tolerance to cow's milk is associated with decreased epitope binding by IgE and a concurrent increase in corresponding epitope binding by IgG4.

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Corresponding author: Hugh Sampson, The Division of Pediatric Allergy and the Jaffe Institute for Food Allergy, Mount Sinai School of Medicine, PO Box 1198, New York, NY 10029-6574, Phone: 212-241-5548, Fax: 212-241-7153, hugh.sampson@mssm.edu.

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Clinical implications

Temporal changes in the cow's milk epitope binding profile of IgE and IgG4 combined may help in predicting the clinical course of cow's milk allergy.

Keywords

cow's milk allergy; tolerance; epitope; IgE; IgG4; IgA

Introduction

Cow's milk allergy (CMA) affects 2-3 % of infants (1,2). Patients are typically sensitized to several cow's milk (CM) proteins. The four proteins in the casein fraction (α_{s1} -, α_{s2} -, β - and κ -casein) as well as α -lactalbumin and β -lactoglobulin are considered major allergens (3). Both conformational and sequential epitopes elicit antibody responses (3,4).

CMA often resolves before school age (1,5,6). High cow's milk (CM) specific IgE levels (6,7) and a strong reaction in CM skin prick testing (5) predict persistence of CMA. Nowak-Węgrzyn et al reported that tolerance of extensively heat-denatured CM was associated with early recovery from CMA (8).

Epitope profiling of IgE antibodies has provided further insight into the relationship between antibody responses and clinical reactivity in CMA. Although the pattern of IgE epitope recognition varied remarkably between individual patients with CMA (9), patients with persistent CMA recognized a wider variety of sequential IgE-binding epitopes compared to patients who recovered from CMA early (10,11). Furthermore, IgE recognition of certain sequential epitopes, especially in the casein fraction, was associated with persistent CMA (10-12). Knowledge on the temporal evolution of IgE epitope recognition is lacking. Of special interest is what happens during the development of tolerance, and how the temporal epitope recognition patterns differ between patients who recover early compared to those with persisting CMA.

Currently, little is known about the epitope recognition by IgG4 and IgA antibodies in food allergies. Both antibody classes are implicated in the development of tolerance to allergens. Several studies have reported that patients undergoing successful aeroallergen specific immunotherapy had increasing levels of specific IgG4 (13-15). Studies on food allergen specific immunotherapy have reported similar phenomena with desensitization to CM (16) or to peanut (17). During successful aeroallergen specific immunotherapy, a few studies have also observed increasing allergen specific IgA (15) or IgA2 levels (18). Furthermore, the natural development of tolerance in egg allergy was associated with an increase in ovalbumin specific IgG4 levels and a decrease in specific IgE levels (19). Both specific IgG4 (20) and IgA (21) may also contribute to the maintenance of clinical tolerance.

We hypothesized that the epitope recognition patterns of IgE, IgG4 and IgA antibodies and their temporal changes would differ between patients with early recovery or persisting CMA, and that the findings could expand our understanding of tolerance development in CMA.

Methods

Study population

We examined serum samples from 23 children with CMA, collected at three time points (Table I), and from 6 non-atopic control subjects at follow-up (mean age 8.6 years, range 8.1-9.3 years). The study population was part of a cohort of 6,209 full-term infants who were prospectively followed for the emergence of CMA (2,5). We had access to clinical data from previous studies (2,5,22). The diagnosis of CMA was confirmed in 118 infants based on an open oral CM challenge after a successful elimination period (2). Patients visited an

outpatient clinic every 6 to 24 months until recovery, which was defined as regular consumption of CM before the visit or as having a negative open oral CM challenge. A follow-up study of patients' CMA status and other atopic manifestations included 94 (80%) of the original population (5). For the current study, we selected 11 patients (6 male) who still had positive CM challenges at a mean age of 8.6 years ("persisting CMA"), and 12 patients (6 male) who had recovered from CMA by age 3 years ("early recovery") (Table I). The selection criteria for the patients were the following: IgE-mediated CMA (Table I), active CMA at mean age 8.6 years (persisting CMA) or recovery by age 3 years (early recovery), and serum samples available at all three time points. CMA was classified as IgE-mediated if the CM specific skin prick test (wheal diameter ≥ 3 mm greater the negative control) or CM specific IgE antibodies (≥ 0.7 kU_A/L measured with UniCAP, Phadia, Uppsala, Sweden) or both were positive at any time point 0-12 months after diagnosis (5). β -lactoglobulin and casein specific IgG4 and IgA levels measured with an enzyme linked immunosorbent assay (23) are shown in Table E1 in the Online Repository.

All patients had skin symptoms at the first, diagnostic CM challenge. Among the 11 subjects with persisting CMA, 10 had urticaria and 1 had eczema. Subjects with early recovery showed urticaria in 10 and eczema in 2 cases. In addition, two children with early recovery vomited immediately after the CM challenge. Two subjects in both study groups had symptoms of the upper respiratory tract; no anaphylactic reactions occurred. We also included 6 non-atopic controls from the follow-up study based on a total IgE level less than 80 kU/L, no history of atopic symptoms and negative skin prick tests with a panel of 18 allergens, described previously (5).

Serum samples were stored at -80°C until analyzed.

Peptide microarray based immunoassay

The peptide microarray-based immunoassay was performed as previously described (24,25) with minor modifications. A library of peptides, consisting of 20 amino acids overlapping by 17 (3-offset), corresponding to the primary sequences of α_{s1} -, α_{s2} -, β -, and κ -caseins, and β -lactoglobulin, was commercially synthesized. Peptides were resuspended in DMSO at 2 mg/mL, diluted 1:2 in Protein Printing Buffer (PPB, TeleChem International, Inc., Sunnyvale, CA) with 0.02% Sarkosyl to a final concentration of 1 mg/mL and printed in two sets of triplicates on epoxy-derivatized glass slides (SuperEpoxy Substrate, TeleChem International, Inc.) using the NanoPrint™ Microarrayer 60 (TeleChem International, Inc.). PPB alone was used as negative control and for background normalization.

The printed slides were blocked with 400 μ L of 1% human serum albumin (HSA) in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) for 60 minutes at room temperature, followed by incubation with 250 μ L of patient serum diluted 1:5 in PBS-T/HSA for 24 hours at 4°C.

For IgE and IgG4 detection, slides were incubated for 24 hours at 4°C with a cocktail of four monoclonal antibodies. Three were monoclonal biotinylated anti-human IgE: one from Invitrogen, Carlsbad, CA, USA and diluted 1:250, one from BD Biosciences Pharmingen, San Jose, CA, USA and diluted 1:250, and one as a gift from Phadia, Uppsala, Sweden, biotinylated in our laboratory and diluted 1:1000. The cocktail further included one monoclonal anti-human IgG4-FITC (Clone: HP6025, Southern Biotechnology Associates Inc., Birmingham, AL), USA) diluted 1:1000. Slides were subsequently washed with PBS-T, incubated for 4 minutes with ethylene diamine tetraacetic acid (EDTA) 1 mM in PBS-T, washed again with PBS-T, equilibrated for 1 minute with Dendrimer Buffer (Genisphere, Hatfield, PA) followed by incubation for 3 hours at 31 °C with a cocktail of Anti-Biotin-Dendrimer_Oyster 550 (350) (Genisphere) and Anti-FITC_Dendrimer_Oyster 650 (350)

(Genisphere) in Dendrimer Buffer both at 0.6 µg/ml with addition of 0.02 µg/ml of salmon sperm DNA. Finally, slides were washed with PBS-T, 15 mM Tris buffer, centrifuge dried, followed by wash with 0.1 X PBS, centrifuge dried, washed again with 0.05 X PBS and centrifuge dried.

For IgA detection, following serum incubation and washing, slides were incubated for 1 h at 31 ° C with polyclonal goat anti-human IgA diluted 1: 250 (Sigma-Aldrich), which was covalently conjugated with Alexa 546 (Molecular Probes – Invitrogen) according to the manufacturer's instructions. Slides were then washed with PBST and distilled water, and then centrifuge dried. Immunolabeled slides were scanned using a ScanArray®Gx (PerkinElmer, Waltham, MA). Images were saved in TIFF.

Bioinformatic analysis

A novel method for image analysis of the peptide microarray-based immunoassay data was developed. Peptide array chip images were checked for quality, and the spot intensities were quantified as the means of the detected spot area brightness. The local backgrounds of the spots were detected and the normalized value for *i*th spot (I_i) is calculated using control spots (empty spots at the peptide array) as

$$I_i = (I - I_b) / (I_p - I_{pb}), \text{ where}$$

I = spot mean intensity

I_b = spot local background intensity

I_p = control spots' median intensity

I_{pb} = control spot local background intensity

A median of the peptide spot intensities were calculated for each chip. The intensity was labeled as active if the intensity was at least 0.5 times standard deviation of peptide intensities for each antibody (Fig E1 in the Online Repository).

In order to find active peptide regions within a sample group, the active peptide hits were convoluted with a Gaussian curve with $\sigma = 2$ to combine possible near hits together (Fig E2-4 in the Online Repository). Smoothed activation values were averaged over the sample group. The differences between groups and time points were calculated from the smoothed averages. A peptide region was labeled active if at least half of the patients in a group had an active peptide in the region (Fig E5 in the Online Repository).

In order to investigate whether a set of peptides could assign the subjects to correct classes, and thus predict the clinical pace of recovery from CMA, we used a random decision tree algorithm. Decision tree prediction methods are both strong predictors and able to identify interactions between variables, and therefore successfully used in several biomedical applications (26,27). The random decision tree algorithm creates a large number of decision trees and uses them as an ensemble to achieve robust and accurate prediction of performance.

Peptide binding by IgE, IgG4 and IgA was coded dichotomously as active or absent. As 23 samples were observed to be too small to result in robust results (data not shown), we combined IgE with IgG4 and IgA datasets and used the resulting 46 samples in the subsequent analyses. We selected the most informative peptides with a feature selection algorithm that considers peptide relevance and redundancy (28). We performed statistical validation using three-out-cross-validation accompanied with area under (AUC) the receiver operating characteristic curve (ROC), and the κ -value that describes how much the

agreement on classification results differs from random guessing. Feature selection, prediction and statistical validation analyses were conducted with the Weka software (29).

Results

Patients with persisting CMA had more intense and stable IgE peptide binding over time than patients who recovered early

At diagnosis, IgE binding patterns to CM peptides between the two patient groups differed less than at later time points (Fig 1A). Patients with persisting CMA had more intense IgE binding than patients who recovered early in one region on β -casein, three regions in β -lactoglobulin and one wide region on κ -casein (Fig 1A). The recognition profile of patients with persisting CMA did not change much over time (Fig 1 A-D). The signal overall was strongest at the time of diagnosis, except for a region in α s2-casein and one in κ -casein, which gave a stronger signal at follow-up than at earlier time points (Fig 1A-D). In contrast, IgE from patients who recovered early recognized fewer peptides over time (Fig1A-D, Table II) except for an increased signal at follow-up in a region of κ -casein (Fig1C-D). At follow-up, patients with persisting CMA bound to large regions in α s1-casein, whereas only few regions in α s2-casein, β -casein, and κ -casein and none in β -lactoglobulin showed significant binding (Fig 1C). In differences of IgE binding between the time of diagnosis and one year later or at final follow-up, the binding increased in regions of α s1- and α s2-caseins more in patients with persisting CMA than in patients with early recovery (Fig 1E). In regions of β -casein, β -lactoglobulin and κ -casein, the opposite was observed: IgE binding increased more in patients with early recovery compared to those with persisting CMA (Fig 1E). Non-atopic control subjects did not show any significant IgE binding to CM epitopes (data not shown.)

Patients with persisting CMA or early recovery had comparable IgG4 peptide binding patterns

At the time of diagnosis, IgG4 from children with early recovery bound to approximately the same protein regions as IgG4 from children with persisting CMA, but at lower intensity (Fig 2A). Wide regions, especially in α s2-casein and β -casein, remained unrecognized (Fig 2A). Differences emerged primarily due to higher intensity of binding in patients with persisting CMA or due to the two groups recognizing different regions located very close to each other on the same protein (Fig 2A). One region in β -casein, however, was recognized among children with persisting CMA but not in those with early recovery (Fig 2A); the difference was similar with IgE binding in the same region (Fig 1A). The IgG4 binding profiles changed little over time, whereas intensity (reflecting antibody concentration) of the binding signal increased except for the terminal end of κ -casein (Fig 2A-D, Fig E6-E7 in the Online Repository). At follow-up, children with persisting CMA bound peptides more intensely in regions of α s1-casein and β -casein than children with early recovery, whereas a few regions in α s2-casein, β -lactoglobulin and κ -casein showed more intense binding in children with early recovery (Fig 2C-D). Comparing the changes in IgG4 binding from the time of diagnosis and one year later or at the final follow-up, no clear pattern was observed in differences of IgG4 binding between the two groups (Fig 2E).

Peptide binding by IgA increased over time particularly among patients with persisting CMA

IgA binding was low overall (Fig 3, data not shown). It increased, however, at follow-up particularly in children with persisting CMA compared with earlier time points (Fig 3B). At follow-up, both groups had binding with high signal intensity at the terminal end of α s2-casein (Fig 3A-B). The recognition profiles were similar in the two groups, except for one region in α s2-casein, two regions in β -casein and one in β -lactoglobulin where children with

persisting CMA showed higher signal intensity in IgA binding than those with early recovery (Fig 3A). Comparing the changes in IgA binding from the time of diagnosis and one year later, the magnitude of binding in the two groups did not differ initially (data not shown). At the time of follow-up, however, the intensity of IgA binding had increased more in patients with persisting CMA in several regions across all five proteins except for two regions in κ -casein (Fig 3C).

Peptide binding by IgE had overlap with IgG4, but not with IgA

The binding pattern of IgG4 antibodies was similar to that of IgE in both groups (Fig 1, Fig 2). Among children with early recovery, IgE binding decreased over time while IgG4 binding remained at about the same level, or increased in some regions by the time of follow-up (Fig 1, Fig 2, Table II, Fig E6 in the Online Repository). Among children with persisting CMA, the signal intensity of IgE binding remained comparable or became more intense than IgG4 binding, apart from regions in β -casein (Fig 1, Fig 2, Table II, Fig E7 in the Online Repository). At the time of diagnosis, differences between IgE and IgG4 peptide binding were few (Fig E6-E7 in the Online Repository) except for regions in α s1-casein where IgE and IgG4 binding intensity overlapped less in children with early recovery than those with persisting CMA (Fig 4A). The difference between IgE binding intensity and that of IgG4 was greater, indicating less overlap, in children with persisting CMA compared to those with early recovery at two time points following the diagnosis, primarily in regions of α s1- and α s2-caseins; a region in β -casein being an exception at the time of follow-up (Fig 4A).

The binding profiles of IgE and IgA had little overlap in α s1- and α s2-caseins in either group, but had somewhat more in β -casein, β -lactoglobulin and κ -casein (Fig 1A-C, Fig 3A; data not shown). The differences between IgE binding intensity and that of IgA were greater in children with persisting CMA compared to those with early recovery at diagnosis and one year later in regions of α s1- and α s2-caseins (Fig 4B). In contrast, the differences were smaller at follow-up in children with persisting CMA compared to patients with early recovery in regions of α s2- and β -casein, β -lactoglobulin and κ -casein (Fig 4B).

IgE and IgG4 binding at diagnosis to a panel of peptides discerns patients who develop persisting CMA from patients with early recovery

Random decision tree analysis revealed that IgE and IgG4 binding to a panel of regions in α s1-, α s2-, β - and κ -casein (Table III) categorized the two patient groups at the time of diagnosis with significant accuracy (AUC 92 %, κ -statistic 0.87). Sensitivity and specificity of this classification for separating patients with persistent CMA from patients that recovered from CMA were 96% and 91%, respectively. The prediction accuracy did not improve when we included CM IgE level (measured with UniCAP) as a variable to the dataset before and after the feature selection step (data not shown).

Discussion

Our prospective, longitudinal study on the natural course of CMA showed that IgE and IgG4 antibodies recognize similar epitopes on the various CM proteins. The finding supports the hypothesis that IgG4 induces tolerance by blocking the binding of specific IgE to allergen (14,30). We observed that an increase in the intensity of IgG4 binding to CM epitopes occurred concurrently with a decrease in IgE binding intensity among patients who recovered early from CMA. This is consistent with previous observations on levels of antigen specific IgE and IgG4 in the natural course of hen's egg allergy (19), in desensitization to CM (16) or peanut (17) and in successful aeroallergen specific immunotherapy (13-15).

Epitope recognition patterns of IgE and IgA had little overlap. It may reflect the observation that specific IgA, in contrast to IgG4, does not inhibit IgE binding (18,31). The intensity of peptide binding by IgA increased over time in children with persisting CMA, whereas it changed little in children with early recovery. Furthermore, the estimated differences between IgE binding intensity and that of IgA showed no group-associated trend. Our data thus do not fully support the reported role of serum IgA in tolerance development (15,18). A possible technical reason for the low IgA binding signal in α -caseins is that the more sensitive dendrimer amplification was not applied to the IgA assay.

The IgE epitope recognition profile in patients with persisting CMA at the age of 8-9 years was stable over time, whereas patients who recovered by the age of 3 years lost peptide-specific binding activity over time. The observation is consistent with reports that broader epitope profiles are associated with persisting CMA (10,11). The greater intensity of IgE binding signal in patients with persisting CMA is in accordance with results that higher CM specific IgE levels predict prolonged clinical reactivity to CM (6).

IgE binding patterns were similar at the time of diagnosis in both patient groups and thus did not provide prognostic information. However, children with persisting CMA recognized peptide regions in β -casein, β -lactoglobulin and κ -casein with greater intensity than children with early recovery. IgE binding of regions in these proteins have been associated with persisting CMA (11). Previous studies indicated that IgE binding to α -casein epitopes may predict the natural course of CMA (10-12). In our prospective study, α -casein epitopes had no predictive value at the time of diagnosis, but a year later and especially at the time of follow-up, when children in the group of early recovery already tolerated CM, binding intensity was greater in children with persisting CMA. However, the random decision tree analysis revealed that combining IgE and IgG4 binding data at the time of diagnosis on relatively few regions in α s1-, α s2-, β - and κ -casein predicted with significant accuracy whether a child would recover from CMA early or have persisting allergy.

The discrepancies between this and previous studies on IgE epitope recognition may arise from differences in subject selection and characteristics, from the variability of IgE epitope profiles (9), and from divergence in the stages of CMA under investigation. In this prospective study, CMA was diagnosed at a mean age of 7 months, which was on average within four months from the first symptoms (2). The relatively low CM-specific IgE levels therefore reflect the relatively short period of CM sensitization. Furthermore, we investigated samples from the early (at diagnosis and one year after) and later (at mean age 8.6 years) stages of CMA. Subjects in earlier studies (10,12) had considerably higher CM specific IgE levels and more severe symptoms, including anaphylaxis, than children in the current study, and samples were drawn at school age. Cerecedo et al (11) investigated patients with CM-specific IgE levels more comparable to those in the current study, but they compared CMA patients who were reactive or tolerant to CM at a much younger age (median age 2 years, range 2-4 months). Since patients with CMA have the potential of recovery at any age (6), the life-time prognosis of patients with persisting CMA at school age (10,12) (and in the current study) or at toddler age (11) may differ. Differences in methods, allergen sensitivity, and type of statistical analysis may also contribute to the variation between studies.

Our study demonstrates the significance of decreasing IgE recognition of allergen epitopes with a concurrent increase in corresponding IgG4 recognition in the development of allergen tolerance, whereas the role of circulating specific IgA remains unclear. These findings can potentially be utilized to predict prognosis and to develop novel immunotherapeutic modalities for the treatment of food allergies.

Capsule summary

Cow's milk epitope binding by IgE is stable over time in children with persisting cow's milk allergy, whereas it decreases in those who recover early. Binding patterns by IgE overlap with IgG4, but not IgA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CMA	cow's milk allergy
CM	cow's milk
PPB	protein binding buffer
AUC	area under curve
ROC	receiver operating characteristic curve

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FIG 1.

Differences in IgE binding to cow's milk peptides (black curve) between children with persisting cow's milk allergy at the age of 8-9 years (P, red) and those recovering early by the age of 3 years (E, green) at three time points: A) T:0 the time of diagnosis, B) T:1 one year later, C) T:9 follow-up at the age of 8-9 years. Arrows point to regions with significant (>0.1 or <-0.1) difference between groups. The x-axis depicts amino acid sequences of the five proteins (β -LG stands for β -lactoglobulin), and the y-axis relative signal strength. The temporal differences in IgE binding intensity are shown D) within study groups and E) between groups; red denotes regions with significantly (>0.1) increased and blue regions with significantly (<-0.1) decreased binding intensity.

FIG 2.

Differences in IgG4 binding to cow's milk peptides (black curve) between children with persisting cow's milk allergy at the age of 8-9 years (P, red) and those recovering early by the age of 3 years (E, green) at three time points: A) T:0 the time of diagnosis, B) T:1 one year later, C) T:9 follow-up at the age of 8-9 years. Arrows point to regions with significant (>0.1 or <-0.1) difference between groups. The x-axis depicts amino acid sequences of the five proteins (β -LG stands for β -lactoglobulin), and the y-axis relative signal strength. The temporal differences in IgG4 binding intensity are shown D) within study groups and E) between groups; red denotes regions with significantly (>0.1) increased and blue regions with significantly (<-0.1) decreased binding intensity.

FIG 3.

A) Differences in IgA binding to cow's milk peptides (black curve) between children with persisting cow's milk allergy at the age of 8-9 years (P, red) and those recovering early by the age of 3 years (E, green), at the time of follow-up at the age of 8-9 years (T:9). Arrows point to regions with significant (>0.1 or <-0.1) difference between groups. The x-axis depicts amino acid sequences of the five proteins (β -LG stands for β -lactoglobulin), and the y-axis relative signal strength. The temporal differences in IgA binding intensity are shown B) within study groups and C) between groups; red denotes regions with significantly (>0.1) increased and blue regions with significantly (<-0.1) decreased binding intensity.

FIG 4.

Differences in overlap of antibody binding intensity to cow's milk peptides between children with persisting cow's milk allergy at the age of 8-9 years (P) and those recovering early by the age of 3 years (E). Time points are denoted as follows: T0 the time of diagnosis, T1 one year later, T9 follow-up at the age of 8-9 years. Red denotes regions where the difference of binding intensity between IgE and IgG4 (4A) or between IgE and IgA (4B) is greater (less overlap) in group P compared with group E, and blue denotes the opposite. Differences >0.1 or <-0.1 are considered significant.

Table I

Age, the outcome of an open oral cow's milk challenge and cow's milk specific IgE levels ($\text{kU}_\text{A}/\text{L}$), at three time points in the study groups: patients who had recovered from cow's milk allergy (CMA) by age 3 years, and those with persisting CMA at age 8-9 years

	Recovery from CMA by 3 y	Persisting CMA at age 8-9 y
	age mean (range)	
at diagnosis	7 m (4-12)	7 m (6-12)
1 y after diagnosis	19 m (16-23)	19 m (17-23)
at follow-up	8.5 y (8.0-9.1)	8.6 y (8.1-9.0)
	CM challenge positive/negative (n)	
at diagnosis	12/0	11/0
1 y after diagnosis	6/6	11/0
at follow-up	0/12	11/0
	CM IgE levels kU/L mean (range)	
at diagnosis	4.9 (0.71-14.8)	12.5 (0.18-43.3)
1 y after diagnosis	1.9 (0.2-7.9)	17.1 (0.51-82.7)
at follow-up	0.6 (0.1-4.5)	24.6(0.39-170)

Table II

Increase and decrease in IgE and IgG4 binding as total length of bound cow's milk peptides and as number of recognized regions (in parenthesis) between time points at diagnosis (T0), 1 year after (T1) and at follow-up (T9) in the study groups: patients who had recovered from cow's milk allergy (CMA) by age 3 years, and those with persisting CMA at age 8-9 years

	Recovery from CMA by 3 y		Persisting CMA at age 8-9 y	
	T1-T0	T9-T0	T1-T0	T9-T0
increased IgE	0 (0)	13 (3)	6 (2)	13 (3)
decreased IgE	31 (4)	32 (4)	16 (4)	4 (2)
increased IgG4	4 (1)	16 (4)	3 (1)	25 (6)
decreased IgG4	12 (2)	6 (2)	20 (4)	12 (2)

Table III

Panel of peptides the IgE and IgG4 binding of which discerns at diagnosis patients with persisting CMA from those recovering early

	α 1-casein	α 2 -casein	β -casein	κ -casein
Amino acids	204-224	425-445	119-139	357-377
	459-479	748-768	255-275	408-428
	731-751	867-887	697-717	697-717
			731-751	799-819
			986-1006	