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

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Background: The dynamics and balance of allergen-specific IgE, IgG4, and IgA binding might contribute to the development of tolerance in patients with cow's milk allergy (CMA). Profiling of antibody binding to cow's milk (CM) protein epitopes might help in predicting the natural history of allergy.

Objective: We sought to investigate differences in IgE, IgG4, and IgA binding to CM epitopes over time between patients with early recovery or with persisting CMA.

Methods: We studied serum samples at the time of diagnosis (mean age, 7 months), 1 year later, and at follow-up (mean age, 8.6 years) from 11 patients with persisting IgE-mediated CMA at age 8 to 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 5 major CM 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 CMA, 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 and 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 CM is associated with decreased epitope binding by IgE and a concurrent increase in corresponding epitope binding by IgG4. (J Allergy Clin Immunol 2010;125:1315-21.)

Key words: *Cow's milk allergy, tolerance, epitope, IgE, IgG4, IgA*

Cow's milk allergy (CMA) affects 2% to 3% of infants.[1,2] Patients are typically sensitized to several cow's milk (CM) proteins. The 4 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 CM-specific IgE levels[6,7] and a strong reaction on CM skin prick testing[5] predict persistence of CMA. Nowak-Wegrzyn et al[8] reported that tolerance of extensively heat-denatured CM was associated with early recovery from CMA.

Epitope profiling of IgE antibodies has provided further insight into the relationship between antibody responses and clinical reactivity in patients with 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 with patients who recovered from CMA early.[10,11] Furthermore, IgE recognition of certain sequential epitopes, especially in the casein fraction, was associated with persisting 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 and those with persisting CMA.

Currently, little is known about the epitope recognition by IgG4 and IgA antibodies in patients with 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 peanut.[17] During successful aeroallergen-specific immunotherapy, a few studies have also observed increasing allergen-specific IgA[15] or IgA2[18] levels. Furthermore, the natural development of tolerance in patients with 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] might 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

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Supported by Helsinki University Central Hospital Research Funds, the Foundation for Pediatric Research (Finland), grant AI44326 from the National Institute of Allergy and Infectious Diseases, and a grant from the Food Allergy Initiative.

Disclosure of potential conflict of interest: E. M. Savilahti has received research support from the Foundation for Pediatric Research, Finland, and Helsinki University Central Hospital Research Funds. E. Savilahti has received research support from Helsinki University Central Hospital Research Funds. H. A. Sampson is a consultant for and holds shares in Allertein Pharmaceuticals, LLC; has received research support from the Food Allergy Initiative and the National Institute of Allergy and Infectious Diseases/National Institutes of Health; is a consultant/scientific advisor for the Food Allergy Initiative; and is a part owner of Herbal Springs, LLC. The rest of the authors have declared that they have no conflict of interest.

Received for publication October 30, 2009; revised February 17, 2010; accepted for publication March 15, 2010.

Available online May 12, 2010.

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0091-6749/$36.00

doi:10.1016/j.jaci.2010.03.025

Abbreviations used
CM: Cow's milk
CMA: Cow's milk allergy
PBS-T: PBS containing 0.05% Tween 20

the findings could expand our understanding of tolerance development in patients with CMA.

METHODS

Study population

We examined serum samples from 23 children with CMA collected at 3 time points (Table I) and from 6 nonatopic 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 result. 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 challenge results 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 a mean age of 8.6 years (persisting CMA) or recovery by age 3 years (early recovery), and having serum samples available at all 3 time points. CMA was classified as IgE mediated if the CM-specific skin prick test response (wheal diameter ≥3 mm larger than that elicited by the negative control), CM-specific IgE antibody levels (≥0.7 kU_A/L measured with UniCAP [Phadia, Uppsala, Sweden]), or both were positive at any time point 0 to 12 months after diagnosis.[5] β-Lactoglobulin– and casein-specific IgG4 and IgA levels measured with an ELISA[23] are shown in Table E1 (available in this article's Online Repository at www.jacionline.org).

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, 2 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 nonatopic control subjects from the follow-up study based on a total IgE level of less than 80 kU/L, no history of atopic symptoms, and negative skin prick test responses with a panel of 18 allergens, as 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 dimethyl sulfoxide at 2 mg/mL, diluted 1:2 in Protein Printing Buffer (TeleChem International, Inc, Sunnyvale, Calif) with 0.02% Sarkosyl to a final concentration of 1 mg/mL, and printed in 2 sets of triplicates on epoxy-derivatized glass slides (SuperEpoxy Substrate; TeleChem International, Inc) by using the NanoPrint Microarrayer 60 (TeleChem International, Inc). Protein Printing Buffer alone was used as a negative control and for background normalization.

The printed slides were blocked with 400 μL of 1% human serum albumin in PBS containing 0.05% Tween 20 (PBS-T) for 60 minutes at room temperature, followed by incubation with 250 μL of each patient's serum diluted 1:5 in PBS-T/human serum albumin for 24 hours at 4°C.

For IgE and IgG4 detection, slides were incubated for 24 hours at 4°C with a cocktail of 4 mAbs. Three were monoclonal biotinylated anti-human IgE: one from Invitrogen (Carlsbad, Calif) and diluted 1:250; one from BD Biosciences PharMingen (San Jose, Calif) and diluted 1:250; and one as a gift from Phadia biotinylated in our laboratory and diluted 1:1,000. The cocktail further included one monoclonal anti-human IgG4–fluorescein isothiocyanate (clone HP6025; Southern Biotechnology Associates, Inc, Birmingham, Ala) diluted 1:1,000. Slides were subsequently washed with PBS-T, incubated for 4 minutes with 1 mmol/L EDTA in PBS-T, washed again with PBS-T, and 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 salmon sperm DNA. Finally, slides were washed with PBS-T and 15 mmol/L Tris buffer, centrifuge dried, washed with 0.1× PBS, centrifuge dried, washed again with 0.05× PBS, and centrifuge dried.

For IgA detection, after serum incubation and washing, slides were incubated for 1 hour at 31°C with polyclonal goat anti-human IgA diluted 1:250 (Sigma-Aldrich, St Louis, Mo), which was covalently conjugated with Alexa 546 (Molecular Probes–Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Slides were then washed with PBS-T and distilled water and centrifuge dried.

Immunolabeled slides were scanned with a ScanArray Gx (PerkinElmer, Waltham, Mass). Images were saved in TIFF format.

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 the ith spot (I_i) was calculated by using control spots (empty spots at the peptide array) as follows:

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

where I is defined as spot mean intensity, I_b is defined as spot local background intensity, I_p is defined as control spot median intensity, and I_{pb} is defined as 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 the SD of peptide intensities for each antibody (see Fig E1 in this article's Online Repository at www.jacionline.org).

To find active peptide regions within a sample group, the active peptide hits were convoluted with a Gaussian curve, with a σ value of 2 to combine possible near hits together (see Figs E2-E4 in this article's Online Repository at www.jacionline.org). 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 (see Fig E5 in this article's Online Repository at www.jacionline.org). For further information, see the Methods section in this article's Online Repository at www.jacionline.org.

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 are 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. Because 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 leave-3-out cross-validation accompanied with the area under the receiver

TABLE I. Age, outcome of an open oral CM challenge, and CM-specific IgE levels (in kilounits of antigen per liter) at 3 time points in the study groups: patients who had recovered from CMA by age 3 years and those with persisting CMA at age 8 to 9 years

	Recovery from CMA by 3 y	Persisting CMA at age 8-9 y
	Age, mean (range)	
At diagnosis	7 mo (4-12 mo)	7 mo (6-12 mo)
One year after diagnosis	19 mo (16-23 mo)	19 mo (17-23 mo)
At follow-up	8.5 y (8.0-9.1 y)	8.6 y (8.1-9.0 y)
	Positive/negative CM challenge result (n)	
At diagnosis	12/0	11/0
One year 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)
One year 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)

operating characteristic curve 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 2 patient groups differed less than at later time points (Fig 1, *A*). Patients with persisting CMA had more intense IgE binding than patients who recovered early in 1 region on β-casein, 3 regions on β–lactoglobulin, and 1 wide region on κ-casein (Fig 1, *A*). 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 1 in κ-casein, which showed stronger signals at follow-up than at earlier time points (Fig 1, *A-D*). In contrast, IgE from patients who recovered early recognized fewer peptides over time (Fig 1, *A-D*, and Table II) except for an increased signal at follow-up in a region of κ-casein (Fig 1, *C* and *D*). At follow-up, patients with persisting CMA showed binding 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 1, *C*). In differences of IgE binding between the time of diagnosis and 1 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 1, *E*). In regions of β-casein, β-lactoglobulin, and κ-casein, the opposite was observed: IgE binding increased more in patients with early recovery compared with those with persisting CMA (Fig 1, *E*). Nonatopic 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, although at lower intensity (Fig 2, *A*). Wide regions, especially in α_{s2}-casein and β-casein, remained unrecognized (Fig 2, *A*). Differences emerged primarily as a result of higher intensity of binding in patients with persisting CMA or because of the 2 groups recognizing different regions located very close to each other on the same protein (Fig 2, *A*). One region in β-casein, however, was recognized among children with persisting CMA but not in those with early recovery (Fig 2, *A*); the difference was similar with IgE binding in the same region (Fig 1, *A*). 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 2, *A-D*, and see Figs E6 and E7 in this article's Online Repository at www.jacionline.org). 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 2, *C* and *D*). Comparing the changes in IgG4 binding from the time of diagnosis and 1 year later or at the final follow-up, no clear pattern was observed in differences of IgG4 binding between the 2 groups (Fig 2, *E*).

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 3, *B*). At follow-up, both groups had binding with high signal intensity at the terminal end of α_{s2}-casein (Fig 3, *A* and *B*). The recognition profiles were similar in the 2 groups, except for 1 region in α_{s2}-casein, 2 regions in β-casein, and 1 region in β-lactoglobulin where children with persisting CMA showed higher signal intensity in IgA binding than those with early recovery (Fig 3, *A*). Comparing the changes in IgA binding from the time of diagnosis and 1 year later, the magnitude of binding in the 2 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 5 proteins except for 2 regions in κ-casein (Fig 3, *C*).

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 (Figs 1 and 2). Among children with early recovery, IgE binding decreased over time, whereas IgG4 binding remained at about the same level or increased in some regions by the time of follow-up (Figs 1 and 2, Table II, and see Fig E6). 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 (Figs 1 and 2, Table II, and see Fig E7). At the time of diagnosis, differences between IgE and IgG4 peptide binding were few (see Figs E6 and E7), 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 4, *A*). The difference between IgE-binding intensity and that of IgG4 was greater, indicating less overlap in children with persisting CMA compared with

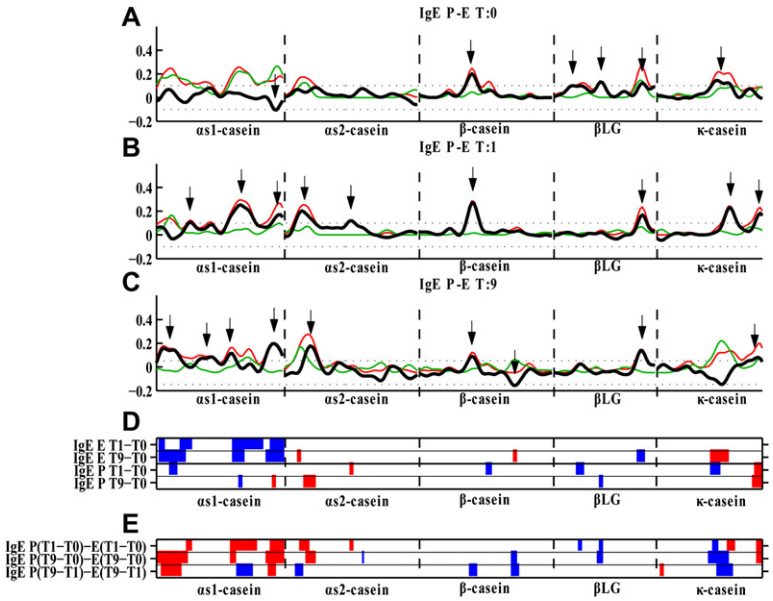

FIG 1. Differences in IgE binding to CM peptides *(black curve)* between children with persisting CMA at the age of 8 to 9 years (*P, red*) and those recovering early by the age of 3 years (*E, green*) at 3 time points: **A**, *T:0,* the time of diagnosis; **B**, *T:1,* 1 year later; **C**, *T:9,* follow-up at the age of 8 to 9 years. *Arrows* point to regions with significant (>0.1 or <−0.1) differences between groups. The *x-axis* depicts amino acid sequences of the 5 proteins, and the *y-axis* depicts relative signal strength. The temporal differences in IgE-binding intensity are shown within study groups **(D)** and between groups **(E)**; *red* denotes regions with significantly (>0.1) increased binding intensity, and *blue* denotes regions with significantly (<−0.1) decreased binding intensity. *β-LG,* β-Lactoglobulin.

TABLE II. Increase and decrease in IgE and IgG4 binding as total length of bound CM peptides and as number of recognized regions (in parentheses) between time points at diagnosis (T0), 1 year later (T1), and at follow-up (T9) in the study groups: patients who had recovered from CMA by age 3 years and those with persisting CMA at age 8 to 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 binding	0 (0)	13 (3)	6 (2)	13 (3)
Decreased IgE binding	31 (4)	32 (4)	16 (4)	4 (2)
Increased IgG4 binding	4 (1)	16 (4)	3 (1)	25 (6)
Decreased IgG4 binding	12 (2)	6 (2)	20 (4)	12 (2)

that seen in those with early recovery at 2 time points after the diagnosis, primarily in regions of α_{s1}- and α_{s2}-caseins, with a region in β-casein being an exception at the time of follow-up (Fig 4, *A*).

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 1, *A-C*; Fig 3, *A*; and data not shown). The differences between IgE-binding intensity and that of IgA were greater in children with persisting CMA compared with differences seen those with early recovery at diagnosis and 1 year later in regions of α_{s1}- and α_{s2}-caseins (Fig 4, *B*). In contrast, the differences were smaller at follow-up in children with persisting CMA compared with those seen in patients with early recovery in regions of α_{s2}- and β-casein, β-lactoglobulin, and κ-casein (Fig 4, *B*).

IgE and IgG4 binding at diagnosis to a panel of peptides discerns patients with 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 2 patient groups at the time of diagnosis with significant accuracy (area under the curve, 92%; κ statistic, 0.87). Sensitivity and specificity of this classification for separating patients with persistent CMA from patients who recovered from CMA were 96% and 91%, respectively. The predictive accuracy did not improve when we included CM IgE level (measured with UniCAP) as a variable in the data set 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. This might 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.

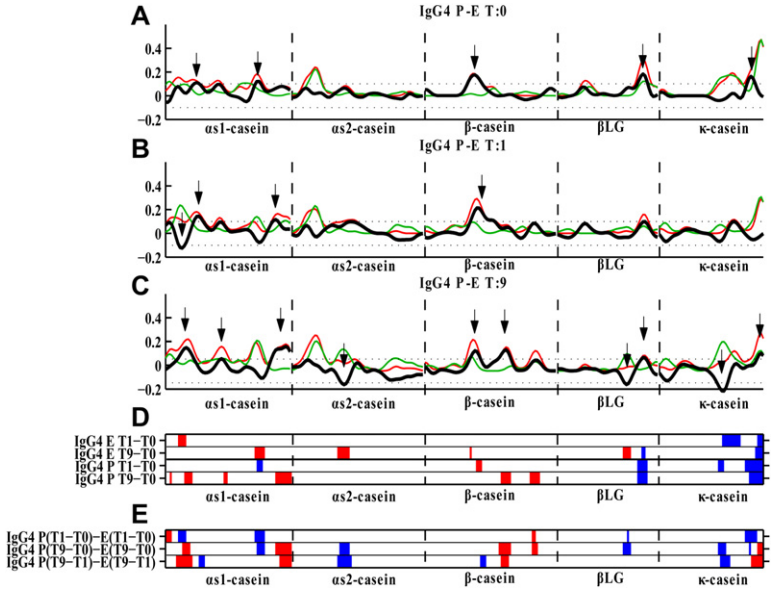

FIG 2. Differences in IgG4 binding to CM peptides *(black curve)* between children with persisting CMA at the age of 8 to 9 years (*P, red*) and those recovering early by the age of 3 years (*E, green*) at 3 time points: **A**, *T:0*, the time of diagnosis; **B**, *T:1*, 1 year later; **C**, *T:9*, follow-up at the age of 8 to 9 years. *Arrows* point to regions with significant (>0.1 or <−0.1) differences between groups. The *x-axis* depicts amino acid sequences of the 5 proteins, and the *y-axis* depicts relative signal strength. The temporal differences in IgG4-binding intensity are shown within study groups **(D)** and between groups **(E)**; *red* denotes regions with significantly (>0.1) increased binding intensity, and *blue* denotes regions with significantly (<−0.1) decreased binding intensity. *β-LG*, β-Lactoglobulin.

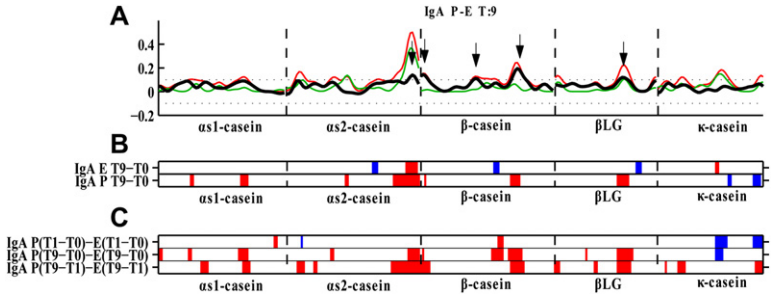

FIG 3. A, Differences in IgA binding to CM peptides *(black curve)* between children with persisting CMA at the age of 8 to 9 years (*P, red*) and those recovering early by the age of 3 years (*E, green*) at the time of follow-up at the age of 8 to 9 years *(T:9)*. *Arrows* point to regions with significant (>0.1 or <−0.1) differences between groups. The *x-axis* depicts amino acid sequences of the 5 proteins, and the *y-axis* depicts relative signal strength. The temporal differences in IgA-binding intensity are shown within study groups **(B)** and between groups **(C)**; *red* denotes regions with significantly (>0.1) increased binding intensity, and *blue* denotes regions with significantly (<−0.1) decreased binding intensity. *β-LG*, β-Lactoglobulin.

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 to 9 years was stable over time, whereas patients who recovered by the age of 3 years lost peptide-specific binding activity over time. This 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 has been associated with persisting CMA.[11] Previous studies indicated that IgE binding to α-casein epitopes might 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 early recovery group already tolerated

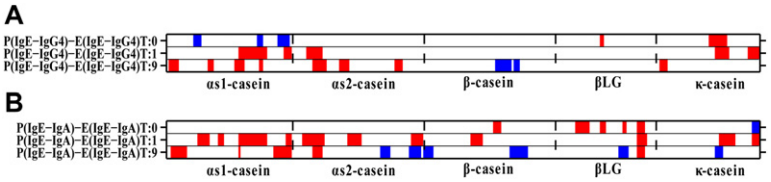

FIG 4. Differences in overlap of antibody-binding intensity to CM peptides between children with persisting CMA at the age of 8 to 9 years *(P)* and those recovering early by the age of 3 years *(E)*. Time points are denoted as follows: *T:0*, the time of diagnosis; *T:1*, 1 year later; *T:9*, follow-up at the age of 8 to 9 years. *Red* denotes regions where the difference of binding intensity between IgE and IgG4 **(A)** or between IgE and IgA **(B)** is greater (less overlap) in group P compared with that in group E, and *blue* denotes the opposite. Differences of greater than 0.1 or less than −0.1 are considered significant. *β-LG*, β-Lactoglobulin.

TABLE III. Panel of peptides with IgE and IgG4 binding that discerns patients with persisting CMA from those recovering early at the time of diagnosis

	α_{s1}-Casein	α_{s2}-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	

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 might 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 4 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 1 year after) and later (at a mean age of 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 with those in the current study, but they compared patients with CMA who were reactive or tolerant to CM at a much younger age (median age, 2 years; range, 2-4 months). Because patients with CMA have the potential for recovery at any age,[6] the lifetime prognosis of patients with persisting CMA at school age (in the current study)[10,12] or at toddler age[11] might differ. Differences in methods, allergen sensitivity, and type of statistical analysis might 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 used to predict prognosis and to develop novel immunotherapeutic modalities for the treatment of food allergies.

We thank Ms Ludmilla Bardina for her technical advice and assistance.

Clinical implications: Temporal changes in the CM epitope–binding profile of IgE and IgG4 combined might help in predicting the clinical course of CMA.

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METHODS

Bioinformatic analysis

The sequential peptides on the microarray-based immunoassay are comprised of 20 amino acids with an offset of 3 amino acids corresponding to the primary sequences of α_{s1}-, α_{s2}-, β-, and κ-casein and β-lactoglobulin. Antigenic peptides usually have a length of at least 6 to 8 amino acids.[E1,E2] Based on this, we chose to smooth the peptide recognition patterns of individual patients.

The signal strength of an active peptide was set at 1 (Fig E1). The discrete Gaussian curve we applied had a mean of 0 and an SD σ value of 2, meaning that 99% of its mass was within 5 steps from 0. When convolving the single active signal with the Gaussian curve, the signal spread to its neighborhood, leaving a value of 0.2 at the location of the active peptide and a value of 0.0088 at the distance of 5 peptides (Fig E2). Active peptides closer than 5 peptides contributed to the signal strength by increasing it (Figs E3-E4).

A single active peptide was considered real if most of the individual study subjects shared it. We set the limit to 50% of the subjects: if the average of smoothed signal strength values among subjects was greater than 0.1, it was considered active throughout the study group (Fig E5). In a similar fashion, if a signal strength difference between 2 groups at a certain location exceeded 0.1, the majority of subjects had a different peptide profile at the location in question.

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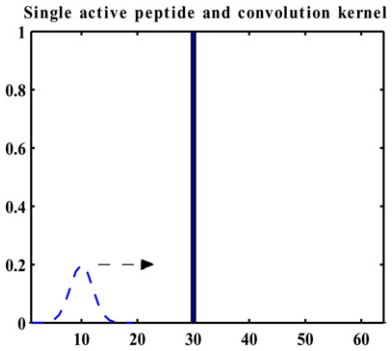

FIG E1. A theoretical situation in which only a single peptide is active. The Gaussian convolution kernel is shown as a *dashed line*.

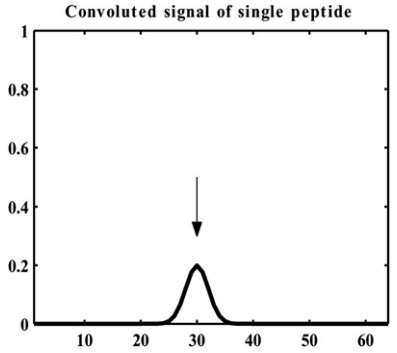

FIG E2. The signal of one single active peptide after convolution with a Gaussian function.

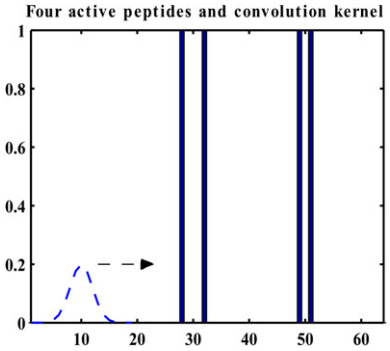

FIG E3. A theoretical situation where four peptides are active. The Gaussian convolution kernel is shown as a dashed line.

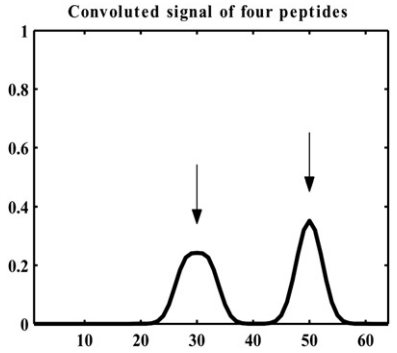

FIG E4. The signal of four active peptides after convolution with a Gaussian function. Note that the peptides closer to each other form a higher signal.

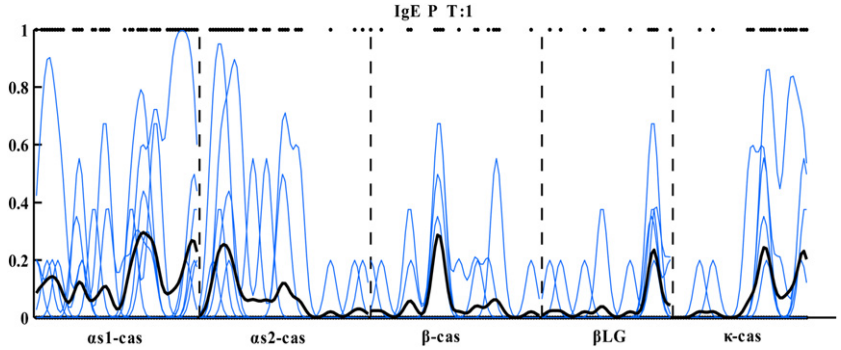

FIG E5. The individual sample signal strengths after convolution *(thin blue line)* and their averages *(thick black line)*.

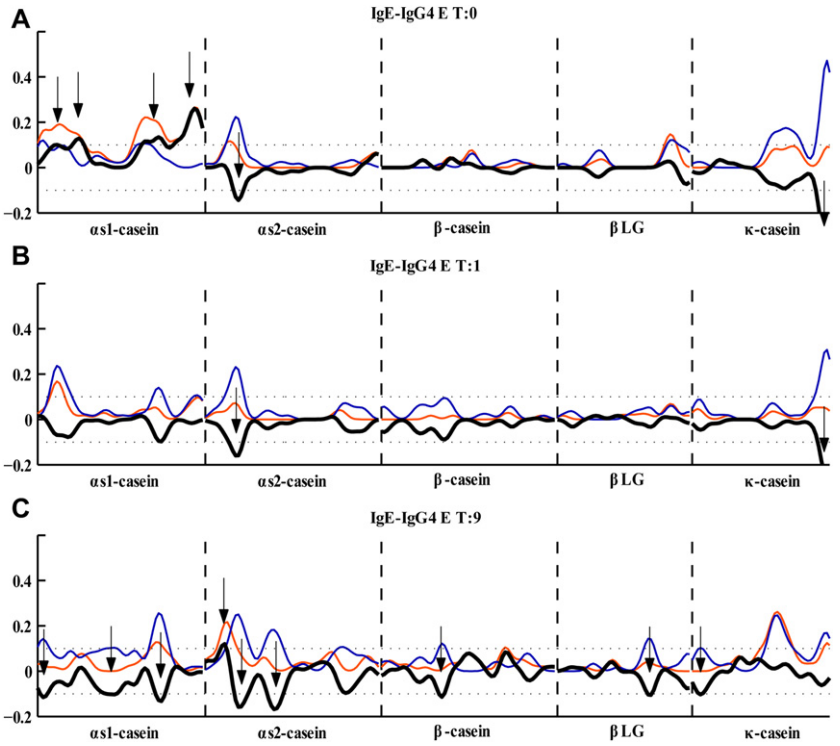

FIG E6. Differences *(black curve)* between IgE *(orange)* and IgG4 *(blue)* binding to CM peptides in children recovering early by the age of 3 years from CMA at 3 time points: **A**, *T:0*, the time of diagnosis; **B**, *T:1*, 1 year later; **C**, *T:9*, follow-up at the age of 8 to 9 years. *Arrows* point to regions with significant (>0.1 or <−0.1) differences between IgE and IgG4 binding. The *x-axis* depicts amino acid sequences of the 5 proteins, and the *y-axis* depicts relative signal strength. *β-LG*, β-Lactoglobulin.

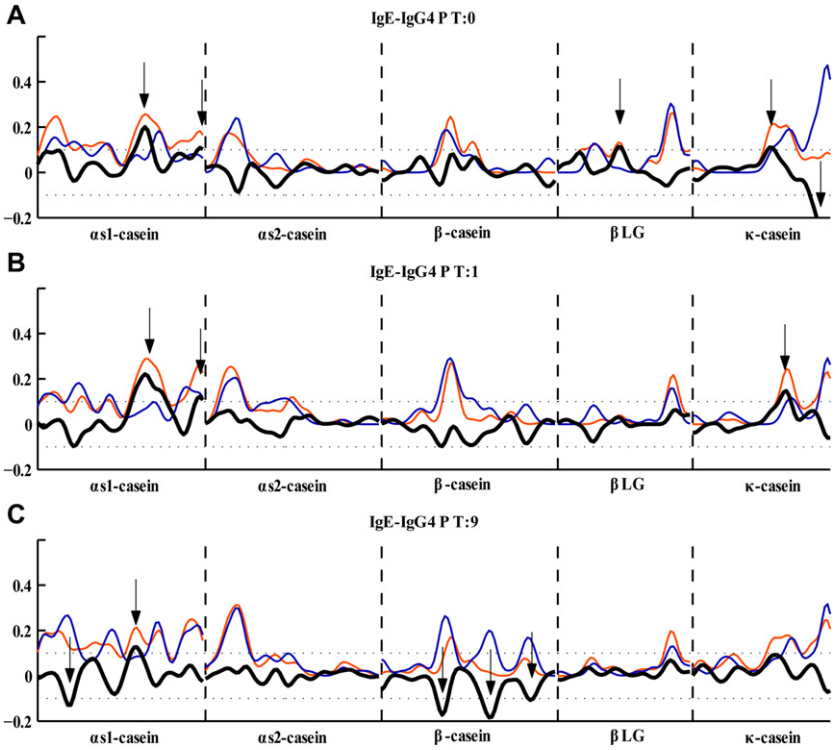

FIG E7. Differences *(black curve)* between IgE *(orange)* and IgG4 *(blue)* binding to CM peptides in children with persisting CMA at the age of 8 to 9 years at 3 time points: **A**, *T:0*, the time of diagnosis; **B**, *T:1*, 1 year later; **C**, *T:9*, follow-up at the age of 8 to 9 years. *Arrows* point to regions with significant (>0.1 or <−0.1) differences between IgE and IgG4 binding. The *x-axis* depicts amino acid sequences of the 5 proteins, and the *y-axis* depicts relative signal strength. *β-LG*, β-Lactoglobulin.

TABLE E1. β-lactoglobulin– and casein-specific IgG4 and IgA levels (in arbitrary units) at 3 time points in the study groups: patients who had recovered from CMA by age 3 years and those with persisting CMA at age 8 to 9 years

	Recovery from CMA by 3 y	Persisting CMA at age 8-9 y
	β-lactoglobulin IgG4 (AU), mean (range)	
At diagnosis	0.08 (0.0-0.2)	0.3 (0.0-1.6)
One year after diagnosis	0.5 (0.0-3.0)	0.4 (0.0-1.2)
At follow-up	2.9 (0.0-16)	0.4 (0.0-1.4)
	β-lactoglobulin IgA (AU), mean (range)	
At diagnosis	0.8 (0.1-3.9)	0.3 (0-1.1)
One year after diagnosis	0.3 (0.0-1.0)	0.3 (0-0.8)
At follow-up	0.06 (0.0-0.4)	0.1 (0-0.3)
	Casein IgG4 (AU), mean (range)	
At diagnosis	0.6 (0.0-2.9)	4.3 (0.0-18)
One year after diagnosis	3.9 (0.0-17)	2.4 (0.0-11)
At follow-up	7.2 (0.0-35)	2.1 (0.0-8.3)
	Casein IgA (AU), mean (range)	
At diagnosis	1.2 (0.4-2.4)	4.5 (0.1-15)
One year after diagnosis	3.3 (0.6-13)	4.3 (0.7-14)
At follow-up	8.5 (0.7-80)	3.8 (0.6-8.2)