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Changes in IgE and IgG4 epitope binding profiles associated with the outcome of oral immunotherapy in cow's milk allergy

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Abstract

Background—Oral immunotherapy (OIT) with cow's milk (CM) has been reported to induce a number of specific antibody responses, but these remain to be fully characterized. Our objective was to explore whether IgE and IgG4 epitope binding profiles could predict the risk of side effects during cow's milk OIT.

Methods—The study population consisted of 32 children (6 – 17 years of age) with cow's milk allergy: 26 children who successfully completed OIT and 6 children who discontinued therapy due to adverse reactions. We investigated sera drawn before and after OIT. We analyzed specific IgE and IgG4 binding to CM protein derived peptides with a microarray based immunoassay. Antibody binding affinity was analyzed with a competition assay where CM proteins in solution competed with peptides printed on the microarray.

Results—IgE binding to CM peptides decreased and IgG4 binding increased following the OIT in children who attained desensitization. Compared with children who successfully completed OIT, those who discontinued OIT due to adverse reactions developed increased quantities and affinity of epitope-specific IgE antibodies and a broader diversity of IgE and IgG4 binding, but less overlap in IgE and IgG4 binding to CM peptides.

Conclusions—Detailed analysis of IgE and IgG4 binding to CM peptides may help in predicting whether CM OIT will be tolerated successfully. It may thus improve the safety of the therapy.

Keywords

antibody affinity; cow's milk allergy; epitope; IgE; IgG4; oral immunotherapy; peptide

Introduction

The use of oral immunotherapy (OIT) with milk proteins may provide a specific and curative therapy for patients with persistent cow's milk allergy (CMA) (1, 2). Studies indicate that CM OIT leads to desensitization in the majority of patients (3–11). In order to improve the safety of OIT, methods for assessing the risk of adverse reactions are needed. We hypothesized that analyzing the diversity and affinity of IgE and IgG4 binding to epitopes on CM proteins may provide one such method, given that profiling such epitope binding correlates with severity of allergic symptoms and the natural development of tolerance in CMA (12–14).

Methods

Study population and cow's milk oral immunotherapy

The University of Helsinki ethics committee approved this study. Children and one of their parents gave their written informed consent.

We recruited children 6 – 17 years of age who had CMA and attended the out-patient clinic at the Hospital for Allergic and Skin Diseases, University of Helsinki. Children included in the study had an immediate reaction to CM in a controlled open oral challenge and at the time of the oral challenge either CM IgE > 5 kU_A/l, or CM IgE >1.5 kUA/L plus a CM SPT wheal size > 5 mm, and serum samples available both at the initiation and completion of OIT.

At diagnosis patients had experienced skin symptoms, erythema, urticaria, some patients had oral pruritus. The majority (29/32) had undergone an open oral milk challenge within 3 months prior to starting OIT. Three subjects who had undergone an open oral milk challenge earlier had experienced a convincing immediate reaction to CM within 3 months prior to starting OIT. The most common symptoms observed during oral CM challenges were the following: laryngopharyngeal pruritus and/or edema in 14/26 of those who successfully completed OIT and in 2/6 of those who discontinued OIT; cutaneous symptoms in 12/26 and 3/6; and gastrointestinal symptoms in 5/26 and 3/6 of patients who completed OIT successfully and those who discontinued therapy, respectively. Symptoms involving more than one organ system were seen in 14/26 and 3/6 of in these two groups, respectively. No severe anaphylactic reactions occurred.

The children and their parents filled out a symptom questionnaire before, immediately after and 3 months after starting OIT. CM SPTs were performed and serum samples taken prior to and following OIT. We measured CM specific IgE antibodies in sera with an enzymatic fluorescent immunoassay, Pharmacia CAP system (Pharmacia Ltd, Uppsala, Sweden) and CM specific IgG4 levels with an enzyme-linked immunosorbent assay (ELISA) described in Savilahti et al, 2010 (15).

Cow's milk oral immunotherapy

CM OIT doses were escalated gradually (Table E1 in the electronic repository). In case of respiratory infection, diarrhea or fever, or considerable adverse reactions in the previous

weeks, the dose increase was postponed for one week when the dose escalation was continued according to the protocol (Table E1). Patients were given 10 mg of cetirizine once daily throughout the OIT protocol and for 2 weeks following the completion of OIT. They were prescribed adrenaline auto-injectors, antihistamine and corticosteroid tablets for use in case of adverse reactions. Compliance with OIT was checked at each appointment with a study physician or nurse. If adverse symptoms clearly compromised the patient's quality of life and continued despite several adjustments to the dosing, the physician together with the patient and family would conclude that OIT should be discontinued.

Children continued daily intake of CM at the maintenance CM dose (usually 200 ml/day corresponding to approximately 7.5 g of proteins) following the termination of OIT. Three months they returned for a follow-up visit. OIT was considered successful when the patient achieved desensitization to CM and could incorporate CM protein into the diet on a daily basis. OIT was considered as “failed” in cases where the therapy had to be discontinued or the patient could not tolerate CM in the daily diet due to adverse reactions.

Peptide microarray based immunoassay

The peptide microarray-based immunoassay was performed as previously described (16, 17) with minor modifications, and a competition assay performed as previously described (14). The library of 235 peptides consisted of 20 amino acids overlapping by 17 (3-offset) and corresponded to the primary sequences of α_{s1} -, α_{s2} -, β -, and κ -caseins, and β -lactoglobulin. The method is depicted in the electronic repository.

The competition assay was run only on samples drawn before initiating OIT. We included serum samples from all children who had discontinued OIT (n=6) and 8 selected samples from children experiencing successful OIT and showed the greatest IgE binding with the standard protocol.

Statistical analysis

We used normalized standardizations derived from peptide fluorescence intensities as a base for all of our analyses as described by Wang et al. (14). A standardization value of zero represents the mean background fluorescence. A peptide was considered to show binding and designated as “positive” if its standardization exceeded 3. This conservative value represents a threshold for which 99% of the background values are smaller. For the competition assay, antibody binding score differences in the two incubation conditions (protein solution or buffer only) for each patient were used to reflect the competition effect and thus antibody binding affinity.

Binding frequencies are reported as the percentage of patients in a group with a fluorescence intensity standardization > 3 for the respective peptide. We located peptide areas with a significant difference between groups based on two criteria: at least 30 % difference in binding frequency and a standardization difference that resulted in $p < 0.05$ with Wilcoxon rank sum test for the peptide and also for both its neighboring peptides (14).

For comparisons, we used either two-tailed Wilcoxon rank sum or Wilcoxon signed rank test, where applicable. As we sought methods to distinguish children who discontinued OIT

due to adverse effects and children who successfully completed OIT, we also computed overlap adjusted combined IgE and IgG4 binding intensities with

$\Sigma \left(IgE_t - \sqrt{IgE_t \cdot IgG4_t + IgG4_t} \right)$ where i represents ranges over a patient's standardizations for all the peptides in the array and computed competition assay adjusted combined IgE and IgG4 binding intensities with an adjusted version of $\|IgE - IgG4\|^2 = IgE_a \cdot IgE_a - 2IgE_a \cdot IgG4_a + IgG4_a \cdot IgG4_a$ where the subscript a denotes that a standardization obtained from the standard assay has been adjusted with its affinity reading from the competition assay: $adjusted_i = standard_i \cdot competition_i / buffer_i$.

For all analyses and plotting, we utilized the R programming language in conjunction with the bioinformatics workflow framework Anduril (18).

Results

CM OIT elicited significant adverse reactions in 16/26 of children who successfully completed OIT. Cutaneous, gastrointestinal and laryngopharyngeal symptoms were noted, but no severe reactions occurred. Symptoms lead to discontinuation of therapy in 6 children included cutaneous symptoms (3/6), nausea (2/6), hematochezia (1/6) and cough (1/6). Further characteristics of the study population are depicted in Table 1.

Children who discontinued OIT (group D) had greater CM specific IgE levels at the initiation and termination of OIT compared with children who successfully completed OIT (group S) (Table 1). CM specific IgG4 levels were greater in group D than in group S at the start of OIT (T0), whereas at the end levels did not differ significantly between groups (Table 1). Specific IgE levels decreased significantly and CM specific IgG4 increased significantly from the beginning (T0) to the end (T1) of therapy (Table 1). The pattern of temporal change was similar in both groups. Age and duration of OIT did not differ between the two groups (Table 1).

At the onset of OIT (T0), a larger proportion of group D had IgE binding to a broader diversity of peptides, especially in α -s1-casein, and at higher intensity, i.e. greater concentrations, compared to group S (Fig 1A, Table E2 in the electronic repository). The same observation was seen at the termination of OIT (T1) (Fig 1B). IgE binding decreased over time both in group D (Fig 1C) and in group S (Fig 1D).

IgG4 binding at T0 was more heterogeneous and more intense in group D than in group S (Fig 2A, Table E2 in the electronic repository). In group D, IgG4 binding diversity was greatest to peptides of β -casein, but significant antibody binding was seen to peptides of all caseins (Fig 2A). In group S, IgG4 binding to CM peptides was minimal at T0 compared to group D (Fig 2A). Also at T1, a larger proportion of patients in group D had IgG4 binding to more CM peptides than in group S, even though IgG4 binding had increased in group S over time (Fig 2B). From T0 to T1, IgG4 binding in group D showed no clear trend: it increased in areas of α -s1-casein, and decreased in a region of β -casein, but overall did not change appreciably over time (Fig 2C). In group S, IgG4 binding increased overall from T0 to T1, in particular in regions of α -s1-casein and β -lactoglobulin, but the overall diversity of IgG4 binding changed little (Fig 2D).

In both groups, the difference of IgE to IgG4 binding intensity to CM peptides decreased from T0 to T1 (Fig 2E–F, Table E2 in the electronic repository). However, the difference was greater, i.e. IgE binding was more intense than IgG4 in group D than in group S (Fig 2E–F). In group S, the decrease in the difference of IgE to IgG4 binding intensity was most prominent in a region of α -s1-casein (Fig 2F).

Regions of IgE and IgG4 binding over-lapped more in group S than in group D (Fig 3A–B). Thus, IgE and IgG4 binding coincided in the same epitope regions more in group S than in group D (Fig 3A–B). The effect was most evident when binding intensity was high, i.e. when antibody concentrations were more elevated.

We performed a competition assay at T0 in all samples in group D (n=6) and a subset of group S (n=8) that showed the most extensive IgE binding. The aim was to assess the affinity of IgE and IgG4 binding to CM peptides, and investigate whether antibody affinity could discriminate between the two groups in cases where the sample had IgE binding of high intensity. Competition effect was more evident in group S than group D, i.e. patients in group D had CM-specific IgE antibodies of higher affinity (Fig 4A). A competition effect in IgE binding could be observed in a larger portion (5/8) of patients in group S than in group D (2/6) (Fig 4B).

Similarly, the IgG4 binding competition effect was weaker, i.e. affinity was greater, in group D than in group S (Fig 4C). Interestingly, most patients (5/6) in group D showed no competition effect in IgG4 binding, i.e. IgG4 binding affinity was very high. In contrast, in group S, only 2/8 showed no competition effect, i.e. they had lower IgG4 binding affinity (Fig 4D).

Discussion

Our study of children with CMA treated with OIT demonstrated that IgE and IgG4 binding of high intensity to a broad spectrum of CM peptides was associated with failure to complete OIT successfully. Further characteristics for those who failed OIT were high IgE and IgG4 binding affinities to CM peptides with little overlap in IgE and IgG4 binding.

The observed increase in IgG4 binding to CM peptides/epitopes as well as in CM IgG4 levels as a result OIT is in line with previous studies that have measured allergen specific IgG4 levels in CM OIT (7, 8) and peanut OIT (19, 20), and in other allergen-specific immunotherapies (21). IgE binding to CM peptides/epitopes and CM IgE levels concomitantly decreased, which conforms to several previous studies on food OIT (4, 6, 9, 10, 19), although some studies reported no change in specific IgE levels (3, 7, 8). In our current study, OIT induced similar antibody changes to CM protein regardless of whether clinical desensitization was achieved.

Overlap in IgE and IgG4 binding to CM peptides, i.e. IgE and IgG4 antibodies binding to the same epitope, was greater in children who completed OIT successfully than in those who were required to discontinue therapy. This observation conforms to the concept that specific IgG4 acts as a blocking antibody to IgE (22). However, IgG4 binding to whole CM proteins and CM epitopes was greater in children who failed OIT, our results underscore the concept

that the most relevant factor to clinical outcome may be the capacity of IgG4 to bind to same epitopes as IgE (22, 23). In addition, greater initial IgE and IgG4 levels may reflect the failure of inducible T regulatory cells in controlling Th2 activity (24, 25). Moreover, OIT in these patients may not have induced a sufficient shift from IL-4 to IFN- γ production(26).

Based on these findings and another recent study (11), intense epitope-specific IgE binding predicts not only persistence of CMA in untreated patients (15, 27, 28), but also in those undergoing OIT. Furthermore, IgE binding to a wide spectrum of CM peptides appears to predict both the failure to develop natural tolerance (12–14) and the failure to achieve OIT-induced desensitization (current study).

Our study suggests three possible approaches for predicting the success of CM OIT utilizing the peptide microarray immunoassay. First, higher IgE/IgG4 ratios in CM peptide binding predict the likelihood of failing desensitization. Second, IgE and IgG4 peptide binding overlapped less in children who could not tolerate OIT compared with those who successfully completed therapy. Third, patients who discontinued OIT had higher affinity IgE and IgG4 binding to CM peptides.

A major short-coming of our study is the lack of a control group. However, based on previous research data on the natural course of CMA (13) and on control groups of CM OIT studies (6–8), we deem it unlikely that any significant immunological changes would have occurred in this time period in a group of age-matched children with CMA without a therapeutic intervention.

In summary, children who discontinued OIT due to adverse effects, had IgE and IgG4 antibodies that bound to CM peptides with greater intensity, broader diversity and greater affinity than in children who successfully completed OIT. In the latter group, IgE binding decreased and IgG4 binding increased significantly from the initiation to completion of OIT. Moreover, children who successfully completed OIT had IgE and IgG4 antibodies that more often recognized the same epitopes. In children with high IgE binding to CMepitopes, lower antibody affinity may discriminate those who will complete OIT successfully from those who will have to discontinue therapy. Combining measurements of IgE and IgG4 binding intensity and affinity to the CM epitopes distinguishes the two patient groups better than any single traditional parameter (Figure 5).

Our study indicates that detailed analysis of IgE and IgG4 binding to a library of CM peptides is useful in understanding immunological changes during OIT and in revealing immunological differences that are associated with clinical outcome. If further studies on other patient populations confirm these results, novel methods for assessing the risk of adverse reactions could make OIT for food allergy safer in the clinical practice. Evidence-based immunological parameters could assist in the selection of patients for OIT, and OIT protocols could be designed more individually based on the risk assessment of adverse reactions, which would improve safety and patients' compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CM	cow's milk
CMA	cow's milk allergy
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
HSA	human serum albumin
Ig	immunoglobulin
OIT	oral immunotherapy
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing 0.05% Tween 20
PPB	protein printing buffer
SPT	skin prick test

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Figure 1.

IgE binding to a library of peptides derived from 5 cow's milk proteins shown as percentage of patients with significant peptide binding. A, B) Comparison of the two patient groups: children who discontinued cow's milk oral immunotherapy (OIT) due to adverse reactions (red, n=6) and children who successfully completed OIT (blue, n=26) A) at the start (T0) and B) at the end (T1) of OIT. C, D) Comparison of T0 (continuous line) and T1 (dotted line) within patient groups C) in children who discontinued OIT (n=6), D) in children who successfully completed OIT (n=26). The x-axis shows the overlapping peptides on the microarray. The y-axis shows the percentage of patients within each group that showed binding with intensity of standardization >3 to each peptide. Black bars mark peptide areas with a standardization difference that resulted in $p < 0.05$ with Wilcoxon rank sum test and at least 30% difference in binding frequency between groups.

Figure 2.

IgG4 binding (A–D) and the difference of IgE and IgG4 binding (E–F) to a library of peptides derived from 5 cow's milk proteins shown as percentage of patients with significant peptide binding. A, B) Comparison of the two patient groups: children who discontinued cow's milk oral immunotherapy (OIT) due to adverse reactions (red, n=6) and children who successfully completed OIT (blue, n=26) A) at the start (T0) and B) at the end (T1) of OIT. C, D) Comparison of T0 (continuous line) and T1 (dotted line) within patient groups C) in children who discontinued OIT (n=6), D) in children who successfully completed OIT (n=26). E, F) Comparison of the difference of IgE and IgG4 binding within patient groups between the start (T0, continuous line) and the end (T1, dotted line) of cow's milk oral immunotherapy (OIT) within patient groups E) in children who discontinued OIT due to adverse reactions (red, n=6), F) in children who successfully completed OIT (blue, n=26).

The x-axis shows the overlapping peptides on the microarray. The y-axis shows the percentage of patients within each group that showed binding with intensity of standardization >3 to each peptide. Black bars mark peptide areas with a standardization difference that resulted in $p < 0.05$ with Wilcoxon rank sum test and at least 30 % difference in binding frequency between groups.

Figure 3.

Co-localization (i.e. overlap) of IgE and IgG4 binding to a library of peptides derived from 5 cow's milk proteins. Comparison between children who discontinued cow's milk oral immunotherapy (OIT) due to adverse reactions (red, n=6) and children who successfully completed OIT (blue, n=26), and all patients (grey, n=32). The y-axis shows the Pearson correlation of peptide locations bound by IgE and IgG4 in relation to A) IgE binding intensity, B) IgG4 binding intensity on x-axis. High correlation points to overlapping IgE and IgG4 binding.

Figure 4.

Binding affinity of IgE and IgG4 to a library of peptides derived from 5 cow's milk proteins. We ran a competition assay on serum samples drawn at the start (T0) of cow's milk (CM) oral immunotherapy (OIT). We included all samples from children who discontinued OIT due to adverse reactions (red, n=6) and from a selection of children who successfully completed OIT and showed most IgE binding in standard assay (blue, n=8). Competition effect is the difference between antibody binding intensity in competition (incubation with CM protein solution) and reference (incubation with only buffer) conditions. Strong antibody affinity is reflected by little competition effect. A) Competition effect in IgE binding. The x-axis shows the overlapping peptides on the microarray. The y-axis shows the competition effect as the percentage of patients within each group that showed binding with intensity of standardization >3 to each peptide. B) Mean intensity of IgE binding in reference conditions on the x-axis and competition on the y-axis. Study subjects are plotted individually (marked by their ID numbers). C) Competition effect in IgG4 binding. Axes as in A. D) Mean intensity of IgG4 binding in reference conditions on the x-axis and competition on the y-axis. Study subjects are plotted individually (marked by their ID numbers).

Study subjects are plotted individually (marked by their ID numbers).

Figure 5.

Five different methods to distinguish the two patient groups at the start (T0) of cow's milk (CM) oral immunotherapy (OIT): children who discontinued OIT due to adverse reactions (red, n=6) and children who successfully completed OIT (blue, n=26 or 8 for competition assays). Each bar represents an individual patient; patient ID numbers are on the x-axis. A) IgE level to whole CM extract as measured with enzymatic fluorescent immunoassay Pharmacia CAP system, kU_A/L (y-axis). B–E) antibody binding intensity (y-axis) to a library of peptides derived from 5 cow's milk proteins as standardizations: B) IgE binding mean intensity, C) combined IgE and IgG4 binding mean intensities, D) overlap adjusted combined IgE and IgG4 binding mean intensities, E) competition assay adjusted combined IgE and IgG4 binding intensities.

Table 1

Characteristics of the study population.

	CM OIT successful n=26	CM OIT discontinued n=6
age at T0 years, median(range)	9.8 (6–17)	8.7 (6–14)
length of OIT days, median(range)	186 (167–458)	254 (119–387)
side effects in OIT n (%)	16 (62)	6 (100)
CM dose at the end of OIT 200 ml n (%)	23 (88)	1 (17)
CM dose at the end of OIT 100–200 ml n (%)	2 (8)	3 (50)
CM dose at the end of OIT 30 ml n (%)	1 (4)	2 (33)
CM IgE at T0 kU _A /L, geometric mean (95% CI)	11 (7.2–17) ^{*†}	85 (29–256) ^{*†}
CM IgE at T1 kU _A /L, geometric mean (95% CI)	8.0 (4.8–13) ^{*†}	57 (20–167) ^{*†}
CM IgG4 at T0 AU, geometric mean (95% CI)	0.2 (0.08–0.4) ^{*†}	1.9 (0.42–8.8) ^{*†}
CM IgG4 at T1 AU, geometric mean (95% CI)	2.9 (1.0–8.2) [†]	6.5 (0.8–54) [†]

OIT denotes oral immunotherapy, CM cow's milk, T0 is the time at the start of OIT, T1 at the end of OIT. SD denotes standard deviation, AU arbitrary units, CI confidence interval.

* denotes significant (p<0.05) difference between groups based on Mann Whitney U-test.

[†] denotes significant (p<0.05) temporal change tested with general linear model for repeated measures on logarithmic transformations.