

Changes in humoral responses to β -lactoglobulin in tolerant patients suggest a particular role for IgG₄ in delayed, non-IgE-mediated cow's milk allergy

Sletten GBG, Halvorsen R, Egaas E, Halstensen TS. Changes in humoral responses to β -lactoglobulin in tolerant patients suggest a particular role for IgG₄ in delayed, non-IgE-mediated cow's milk allergy. *Pediatr Allergy Immunol* 2006.

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The major cow's milk allergen β -lactoglobulin (β -LG) is relatively resistant to enzymatic degradation and may therefore be involved in non-immunoglobulin (Ig)E-mediated cow's milk allergy (CMA) with delayed gastrointestinal symptoms. Serum levels of β -LG-specific IgG₁, IgG₄, IgE, and IgA were compared in clinically reactive and tolerized IgE-mediated and non-IgE-mediated CMA with delayed gastrointestinal symptoms ($n = 29$) and controls ($n = 10$). Tolerance was associated with decreased β -LG-specific IgE, IgG₁, and IgG₄ levels in both patient groups. However, the significantly increased β -LG-specific IgG₄ levels in clinically reactive non-IgE-mediated CMA patients, and its median 36-fold reduction in tolerant patients, suggested a possible immunopathological role for IgG₄ in delayed CMA. Similarly, the significantly increased β -LG-specific IgE levels in IgE-mediated CMA patients were decreased 44-fold in tolerant patients. The tolerant patients had apparently shifted the humoral immune response from a β -LG-specific IgE- and/or IgG₄-dominated immune response to an IgA-dominated immune response as the IgA/IgE or IgA/IgG₄ ratios increased 90- and 15-fold in tolerant IgE-mediated-, and non-IgE-mediated CMA patients, respectively. Thus, the marked difference in β -LG-specific Ig ratios suggested a tolerance-induced inhibition of a Th₂-type of immune response with significantly increased IgA dominance in both CMA patient groups.

Cow's milk allergy (CMA) is the commonest type of food allergy in young children (< 5 yr) (1). Although a large proportion of these patients become tolerant by the age of three, many continue to have CMA throughout childhood (2). Elevated milk protein-specific IgE is mainly associated with immediate reactions and eczema. A T cell-mediated delayed type of hypersensitivity has been suggested to cause the delayed digestive symptoms (3), which are generally not associated with increased cow's milk protein (CMP)-specific IgE (4, 5).

Although no single milk protein accounts for all IgE-mediated reactions, β -lactoglobulin

(β -LG) appears to be one of the major cow's milk allergens (6). It is relatively resistant to enzymatic degradation and immunogenic protein has, moreover, been detected in the blood circulation after ingestion (7). β -LG may therefore, in particular, be involved in CMA-induced delayed gastrointestinal symptoms.

It has been difficult to show that any CMP-specific immunoglobulin (Ig) levels can predict prognosis or the outcome of cow's milk challenge, even for IgE in IgE-mediated CMA patients (3, 8–10). However, longitudinal studies have revealed that serum levels of CMP-specific IgE decreased during tolerance development (11,

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Key words: cow's milk protein; immunoglobulins

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Accepted 14 March 2006

12). Even though CMP-specific IgG is regarded as a normal physiological response to dietary milk proteins (8), some claim that food-specific IgG is indicative for variants of food allergies. Although this is highly controversial (3, 8, 10, 12, 13) some longitudinal studies have reported that clinically reactive CMA patients had higher β -LG-specific IgG levels than in tolerized patients or controls.

Human IgG consists of four sub-classes, IgG₁₋₄, of which IgG₁ and IgG₄ appear to be the dominating subclasses to food antigens (8). Whereas IgG₁ is complement-fixing and associated with inflammatory systemic Th₁-driven immune reactions, IgG₄ is non-complement-fixing and functionally monovalent (14) and appears to be associated with IgE in a Th₂ immune regulatory fashion [reviewed in (15)].

IgA has a dual protective role in the gastrointestinal mucosa: the dimeric secretory form, sIgA, is secreted into the lumen and exerts luminal antigen exclusion, whereas both monomeric IgA and sIgA in the lamina propria may compete with IgE and IgG for antigen binding and thereby inhibit IgG/IgE-mediated inflammatory pathways (16). It has therefore been suggested that tolerance is associated with increased CMP-specific IgA production (17).

The object of this study was to examine the humoral immune response to β -LG. The inclusion of milk-tolerant children provided an opportunity to examine the changes in immune response in tolerant individuals, especially in non-IgE-mediated CMA with delayed gastrointestinal reactions. β -LG-specific IgG, IgG₁, IgG₄, IgE, and IgA levels were compared in both clinically reactive and tolerized IgE-mediated and non-IgE-mediated CMA patients, and antigen-specific Ig ratios were calculated, as these may better illustrate the overall immunoregulatory events associated with tolerance development.

Materials and methods

Study population

Clinical data and laboratory findings are summarized in Table 1. Children with CMA who were selected for this study had previously been referred by primary practitioners and/or hospital clinics to the Voksentoppen Centre for Asthma and Allergy, the University Hospital specialist clinic. They had a previous history of clinically controlled milk challenge-induced reactions while on a milk-free diet. This initial open milk

challenge was carried out for 3 days in the clinic, and if no response was noted, the milk diet was continued at home for at least a month until a response was evident, and milk-free diet was reintroduced, or tolerance was assumed to have occurred. Immediate symptoms such as itching, urticaria, eczema, asthma, vomiting, abdominal pain, diarrhea, or anaphylaxis were noted. Patients with atopic dermatitis were confirmed challenge positive when milk intake resulted in an immediate exacerbation of the cutaneous symptoms. Delayed symptoms occurred from 24 h to 7–10 days following the start of the challenge period. Follow-up of these patients was done by an experienced clinician (RH), by means of regular telephone contact with the parents, who were instructed in administering the diet and reporting symptoms, such as abdominal pain, loose stools, diarrhea, number of bowel movements or delayed eczematous reactions.

All of the children included, except for four highly reactive IgE-mediated CMA children, were retested as described above, under the supervision of the same clinician, in a clinically controlled open challenge ($n = 27$, median age 7, range 4–13; 10 girls and 17 boys). Two additional tolerant patients (male, 21 and 23 yr), with a clinical history of food allergies including milk and referred to the Norwegian Institute of Public Health for re-assessment of their allergies, were included in the study. The patients were either on a milk-free diet ($n = 23$), consumed milk products ($n = 2$) or were on unrestricted diets ($n = 4$).

The patients were divided into two groups. Patients with a clinical history of IgE-mediated CMA with UniCAP levels above two, with or without milk-exacerbated atopic dermatitis ($n = 15$, median age 8, range 4–23) were separated from patients with milk-induced delayed gastrointestinal reactions ($n = 14$, median age 7, range 4–11). The control group ($n = 10$, median age 38, range 1–64), consisted of two non-allergic, milk-consuming children and eight adults (six females, four males).

Blood samples were obtained prior to challenge. Total and cow's milk-specific IgE serum levels were measured using: the UniCAP Total IgE and the UniCAP Specific IgE FEIA systems (Pharmacia Diagnostics AB, Uppsala, Sweden), with skimmed milk as antigen. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, for CMA patients from the age of 4 only. Enrolment was carried out after informed consent was obtained from the parents.

Table 1. Clinical features of individual patients

Patient	Age (yr)	Gender	Tolerant to milk	Reactions to other food	Total IgE (KU/l)	Specific IgE, (KU/l)	Skin prick test
Group I: IgE-mediated CMA, with/without atopic dermatitis (n = 15)							
MA1	4.4	F	+		598	<0.35	Negative
MA8	12.8	F	—	Egg, fruit	169	<0.35	Negative
MA9	12.4	F	—	Egg, fish	3342	<0.35	Negative
MA10	8.5	M	—	Egg, fish	1500	59.4	+++(+)
MA13	23.2	M	+	Fruit/nuts, pollen	183	<0.35	NA
MA14	21.8	M	+	Fruit/nuts, pollen	9.36	<0.35	NA
MA21	13.2	M	+	Egg, fish	707	0.56	++(+)
MA22	4.0	F	—	Fish	81	10.4	+++(+)
MA23	7.3	M	—	Fish	>5000	78.3	+++
MA25	5.5	M	+	Fish	1300	13.1	++
MA26	10.6	M	—		2017	65.0	++++
MA27	4.6	M	—		>5000	>100	+++
MA28	5.8	M	—		780	47.0	+++
MA30	6.8	F	+	Egg	377	<0.35	Negative
MA31	5.4	M	—		530	87.0	+++
Mean cow's milk-specific IgE, KU/l: 30.86 (<0.35 to >100)							
Group II: non-IgE-mediated gastrointestinal symptoms (n = 14)							
MA2	5.7	M	+		18	<0.35	Negative
MA3	8.1	M	—		<63	<0.35	Negative
MA4	8.2	M	—		<63	<0.35	Negative
MA5	6.4	F	+		6	<0.35	Negative
MA7	7.0	M	—	AD: fish, egg	571	2.0	++
MA11	6.7	M	+	AD: dust mites	40	<0.35	Negative
MA16	7.3	F	+		52	<0.35	Negative
MA17	4.0	F	+		<52	<0.35	Negative
MA18	5.8	F	+		74	<0.35	Negative
MA19	7.1	M	+		8	<0.35	Negative
MA24	10.8	M	—		<70	<0.35	Negative
MA29	10.0	M	—	AD: egg, fish	571	2.0	++
MA32	8.7	M	+		580	0.5	Negative
MA33	4.0	F	+		8	<0.35	Negative
Mean cow's milk-specific IgE, KU/l: 0.60 (<0.35–2)							
Group III: healthy controls (n = 10)							
NC5	4.0	M	+		353	<0.35	Negative
NC6	58	M	+		544	<0.35	—
MA20	0.9	F	+		<2.0	<0.35	Negative
NC7	22	F	+		12.8	<0.35	—
NC8	6	F	+		134	<0.35	—
NC9	53	F	+		2.22	<0.35	—
NC10	60	F	+		6.6	<0.35	—
NC11	37	M	+		<2.0	<0.35	—
NC12	31	M	+		7.9	<0.35	—
NC13	39	F	+	IgE: wheat, pollen	746	<0.35	—

Enzyme-linked immunosorbent assay for β-LG-specific Igs

Immulon 2 HB microtitre plates (ThermoLab-systems, Finland) were coated with β-LG (L-3908, 10 μg/ml for IgG, IgG₁, IgG₄, and IgA, 20 μg/ml for IgE) in 100 μl carbonate/bicarbonate buffer (pH 9.6) (all Sigma-Aldrich, Steinheim, Germany), incubated overnight at 4°C and blocked with PBS containing 1% human serum albumin (Calbiochem, La Jolla, CA, USA), at 37°C for 2 h. Sera from 21 patients with high β-LG-specific IgE titers (UniCAP Specific IgE FEIA) were pooled and used to prepare a standard curve. Serially diluted standard, suitably diluted patient samples (1:10–1:400) or buffer blanks were added in duplicate and

incubated (37°C, 1 h) in a humid atmosphere, washed and followed by either monoclonal anti-human IgG, IgG₄ (IUIS/WHO study panel: clones HP6064/8A4 and HP6011/RJ4, respectively, Skybio, Bedford, UK), IgG₁ (HP6069, Zymed, San Francisco, CA, USA), or rabbit anti-human IgA or IgE (both DakoCytomation, Glostrup, Denmark). The appropriate secondary antibodies were goat-anti-rabbit IgG-HRP (Zymed) or rabbit-anti-mouse IgG-HRP (Dako-Cytomation). Enhanced K-Blue TMB (3,3',5,5'-tetramethylbenzidine dihydrochloride, Neogen, Lexington, KY, USA) was used as substrate for HRP and ODs were read on a VICTOR² 1420 multilabel plate counter (Wallac, Turku,

Finland). Values were expressed as arbitrary units (AU/ml) deduced from the reference serum pool standard curve minus blanks.

SDS-PAGE and immunoblotting

A protein mixture containing equal amounts of β -LG, myoglobin and bovine serum albumin (all Sigma-Aldrich), was run on the Mini-Protein III (Bio-Rad, Hercules, CA, USA) at 200 V on 12% polyacrylamide gel, according to the manufacturer's recommendations. Two lanes from each gel were excised and stained with Coomassie brilliant blue. Separated proteins were electroblotted (70 V, 1 h, RT) on to Protran BA83 0.2 μ m nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and stained with Ponceau S (Sigma-Aldrich). Tris-buffered saline (TBS) containing 0.3% Tween and 3% gelatine (both Sigma-Aldrich) was used as blocking and assay buffer. After blocking for 1 h, 5 mm membranes strips were incubated overnight at 4°C with patient sera (1/10). A serum without detectable IgE to β -LG (CAP < 0.35 KU/l) served as negative control. Human IgE myeloma protein at 250 ng/ml was added to this serum to provide a positive IgE-control. Rabbit anti-human IgE (1.5 h, 1/4000, Dako, Cytomation) was then applied followed by goat anti-rabbit IgG-HRP conjugate (1 h, 1/5000) which was developed with TMB (both Zymed). The band intensities were scored on a semi-quantitative scale in a blind manner from no visible band (0) to intense (5+) staining. Dot blotting of serial dilutions of the IgE myeloma protein and the staining intensity of the positive serum pool was used to calibrate the staining intensity on each blot.

Statistical analysis

Immunoglobulin values were not normally distributed (Shapiro-Wilk *W*-test) so non-parametric tests were applied. Ig values and ratios were compared between patient groups and controls using the Wilcoxon rank sum (Mann-Whitney *U*) test. In all tests, $p < 0.05$ was considered significant. Statistical calculations were performed using the JMP-5.0.1 software package (SAS Inst., Cary, NC, USA).

Methodological considerations

Many CMA patients have multiple symptoms, from skin, gastrointestinal or respiratory tracts (18). Although IgE-mediated reactions are immediate, CMP-induced slow-onset cutaneous or gastrointestinal reactions may occur in patients

with low levels of CMP-specific IgE (4, 19). Grouping patients by symptoms or symptom onset time alone may therefore not discriminate underlying mechanisms. Patients were therefore grouped on the basis of symptoms, onset time and CMP-specific IgE-levels (CAP ≤ 2). All patients with atopic dermatitis and/or immediate reactions to cow's milk were grouped as IgE-mediated CMA, whereas all non-IgE-mediated CMA patients had gastrointestinal reactions occurring more than 24 h after milk ingestion and had CMP-specific CAP ≤ 2 .

Use of the double-blind placebo controlled challenge test (DBPCCT) is considered optimal in this type of study, and should ideally be used. However, DBPCCT demands considerable resources from clinic staff, and is impractical for parents at home. Both the initial open challenge and subsequent rechallenge testing were administered in a specialist University Hospital clinic and followed up by an experienced clinician (RH). Previous testing at the same clinic has revealed that such open challenge is an acceptable alternative for DBPCCT (R Halvorsen, unpublished observations).

The purity of the antigen was confirmed by SDS-PAGE separation and Coomassie brilliant blue staining. The levels of antigen-specific Ig measured by enzyme-linked immunosorbent assay (ELISA) depend on both the efficiency and strength of antigen-binding, i.e., the sum of the total affinities and avidities of the Ig molecules. Examining the β -LG-specific Ig titration ratios may better visualize the overall immune response and type of regulatory events associated with tolerance. Although the presence of high titers of β -LG-specific IgA may have a blocking effect on IgG or its subclasses, the relative distribution of the subclasses has been shown to be relatively unaffected by removal of IgA (20). IgA was therefore not removed from the samples.

In order to rule out the possibility of overlooking unexpectedly high levels of IgG₂ and IgG₃, β -LG-specific pan-IgG levels were compared with the combined β -LG-specific IgG₁ and IgG₄ levels, and found to be highly correlated in IgE-mediated CMA patients ($p < 0.0001$), non-IgE-mediated CMA patients ($p = 0.002$) and controls ($p = 0.003$), apparently excluding any major contribution from these other IgG subclasses.

Results

Patients

Clinically controlled open challenge with cow's milk in 25 of the 29 patients, revealed that 15 of

25 patients were clinically tolerant. The four patients who were not challenged due to risk of severe reactions (MA26, 27, 28, and 31) were considered to be clinically reactive because all had CMP-specific IgE > 47 KU/l, strong CMP-specific skin prick tests (3+) and a clinical history of severe cow's milk reactions. Six of the tolerant patients were in the IgE-mediated CMA group and nine in the non-IgE-mediated CMA group.

Non-IgE-mediated CMA patients had, in particular, higher β-LG-specific IgG₄ levels and lower IgA/IgG₄ ratios than tolerized patients

Reactive non-IgE-mediated CMA patients had 36-fold higher β-LG-specific IgG₄ levels than tolerized patients and nine-fold higher than controls (Fig. 1). All β-LG-specific Ig levels were in fact significantly higher in the clinically reactive non-IgE mediated CMA patients compared with the tolerant patients. However, the markedly decreased IgG₄ level made the median IgG₄/IgG₁ and IgG₄/IgE ratios five-fold lower in tolerized patients, whereas the IgE/IgG₁ ratio did not change (Fig. 2). A similar phenomenon was also observed for the β-LG-specific median IgA/IgG₄ ratio, which was 15-fold higher in

tolerized compared with clinically reactive patients. Although both β-LG-specific IgG₁ and IgA levels were lower in tolerized patients, the IgA/IgG₁ ratio actually increased five-fold ($p < 0.05$) whereas, the IgA/IgE ratio did not increase significantly (Fig. 3).

IgE-mediated CMA patients had, in particular, higher β-LG-specific IgE levels and lower IgA/IgE ratios than tolerized patients

Clinically reactive IgE-mediated CMA patients had 44-fold higher β-LG-specific IgE and nine-fold higher IgG₁ levels than tolerized patients (Fig. 1). In contrast to the non-IgE-mediated patients, the β-LG-specific IgG₄ level was not particularly lower in tolerized IgE-mediated CMA patients ($p = 0.08$). The median IgA/IgE ratio increased 90-fold in tolerized patients, because the β-LG-specific IgA levels were similar in both IgE-mediated patient groups (Fig. 3). A similar change was observed for the IgA/IgG₁ ratio, which increased 23-fold in tolerized patients. The median IgE/IgG₁, IgG₄/IgG₁, and IgG₄/IgE ratios did not change significantly because β-LG-specific IgG₁, IgE, and IgG₄ levels fell to a similar extent in tolerized IgE-mediated CMA patients (Fig. 2).

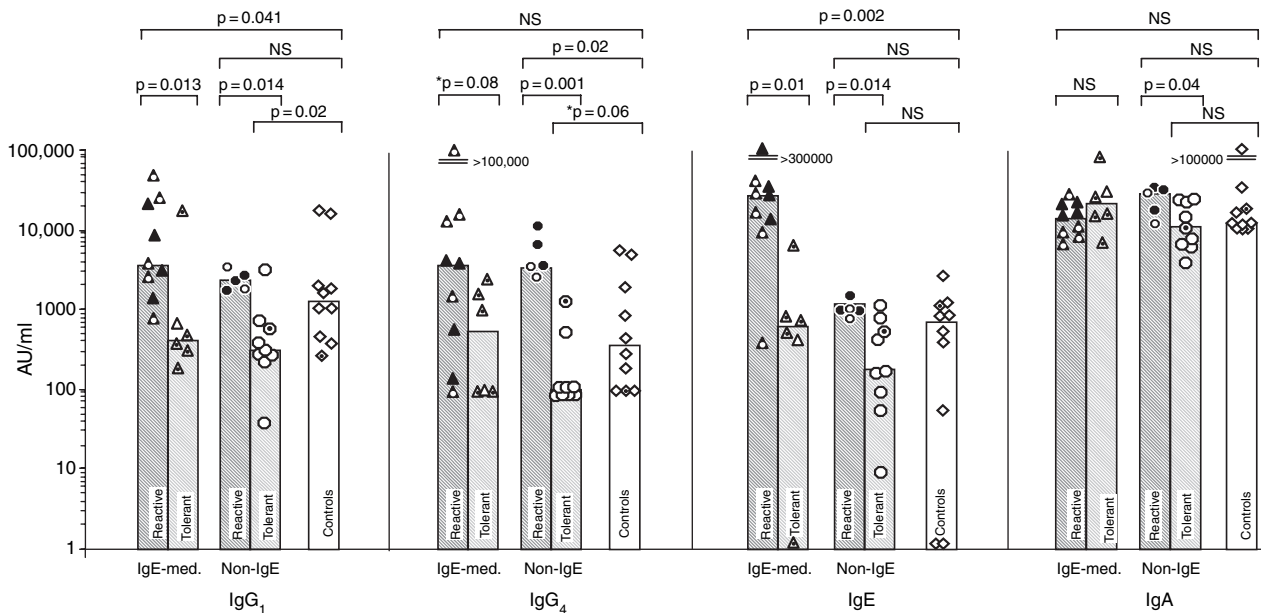


Fig. 1. β-lactoglobulin-specific IgG₁, IgG₄, IgE, and IgA levels in AU/ml (shown using a logarithmic scale) in IgE-mediated and non-IgE-mediated CMA patients. Tolerized non-IgE-mediated patients had markedly lower IgG₄ levels (36-fold) compared with reactive patients. A similar marked difference in IgE levels (44-fold) was seen in tolerized compared with reactive IgE-mediated patients. Tolerized non-IgE-mediated patients had significantly lower IgG₁ and IgG₄ levels than controls, but there were no significant differences between tolerant IgE-mediated CMA patients and controls. Medians are indicated by bars. Reactive patients are represented by closed symbols; tolerant patients by open symbols. Patients reactive to other allergens than cow's milk are marked with a dot within the symbol. Ig values were compared between patient groups and controls using the Wilcoxon rank sum test. $p < 0.05$ was considered significant and * p signified approaching significance.

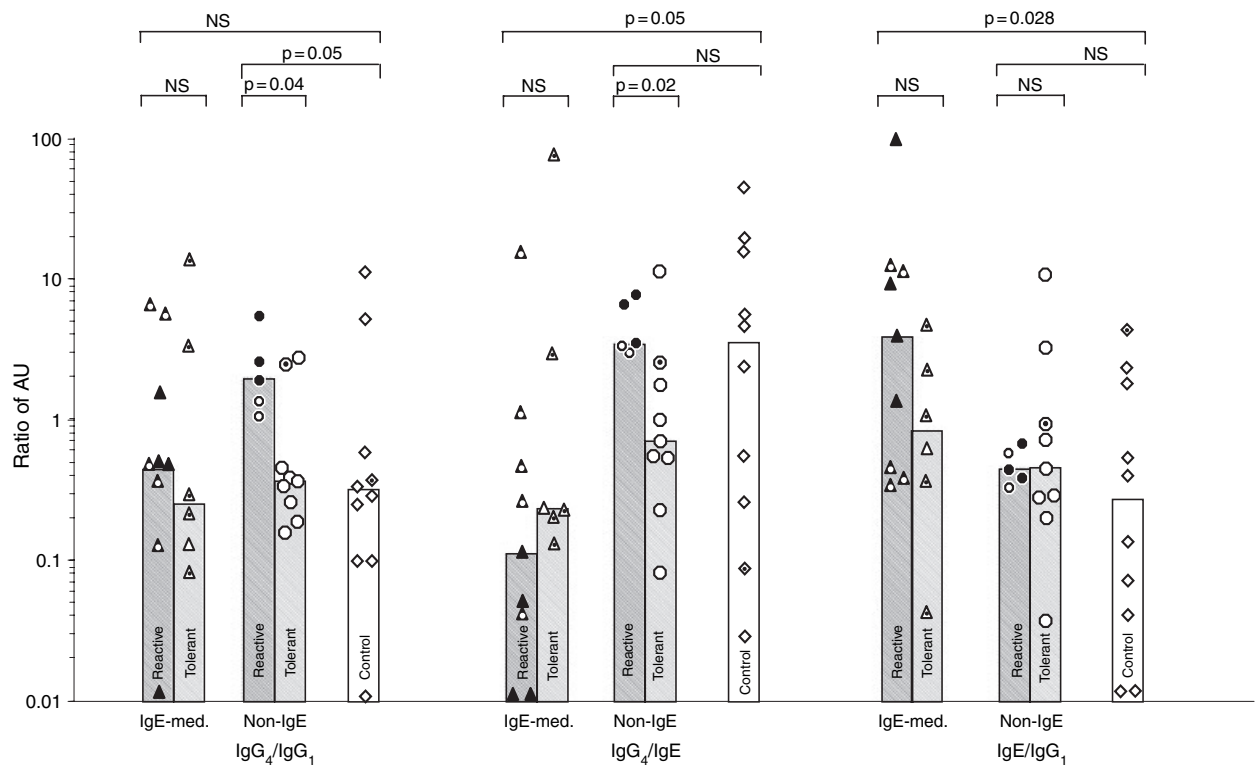


Fig. 2. Ratios of β -lactoglobulin-specific IgG in IgE- and non-IgE-mediated CMA patients: IgG₄/IgG₁, IgG₄/IgE, and IgE/IgG₁ (shown using a logarithmic scale). Median IgG₄/IgG₁ and IgG₄/IgE ratios were significantly lower in tolerantized non-IgE-mediated CMA patients, only. There were no significant differences between tolerant patients and controls, in either patient group. Medians are indicated by bars. Reactive patients are represented by closed symbols; tolerant patients by open symbols. Patients with other allergies than cow's milk are marked with a dot within the symbol. Ratios were compared between patient group and controls using the Wilcoxon rank sum test. $p < 0.05$ was considered significant.

β -LG-specific IgE detected by Western immunoblots

Whereas approximately half of the non-IgE-mediated (8/14) and controls (5/10) had low titers of β -LG-specific IgE on Western blot (median score 1, range 0–3; and 0.5, range 0–3, respectively, not shown), the majority (87%) of the 15 IgE-mediated CMA patients had β -LG-specific IgE scores with significantly higher median scores on the Western blots (median score 2, range 0–4; not shown). Reactive IgE-mediated CMA patients only, had significantly greater IgE scores than tolerant patients ($p = 0.03$). No correlation was found between β -LG-specific ELISA IgE titers and Western immunoblotting IgE scores in either patient group.

Discussion

The present study compared β -LG-specific Ig levels in clinically reactive with tolerant CMA patients, with particular emphasis on non-IgE-associated, delayed-type CMA with delayed gastrointestinal symptoms.

The significantly increased β -LG-specific IgG₄ levels in clinically reactive CMA patients with

non-IgE-associated delayed gastrointestinal symptoms, combined with the lower than normal β -LG-specific IgG₄ levels in tolerantized patients, suggested that IgG₄ is involved in the immunopathological mechanism. Although it was earlier assumed that IgG₄ had a functional role in food allergic individuals (21), it is now considered to be protective, as IgG₄ may compete for antigen binding and has few known inflammatory functions. It may even be functionally monovalent because of exchange of the so-called IgG₄ half-molecules (14). The shift in β -LG-specific IgG₄ may alternatively reflect a generally suppressed systemic immune response to β -LG in tolerantized patients. The latter is supported by the decreased inflammatory Ig (IgG₁ and IgE), as well as IgG₄ levels in tolerantized patients. The IgG₄ subclass is regulated through an IgE-associated Th₂ type of immune response (15). The difference in β -LG-specific IgG₄ in clinically reactive compared with tolerantized patients suggested, therefore, that non-IgE-mediated CMA with delayed gastrointestinal symptoms, rather than being a Th₁-type of delayed hypersensitivity as seen in coeliac disease, is a variant Th₂-type of immune response,

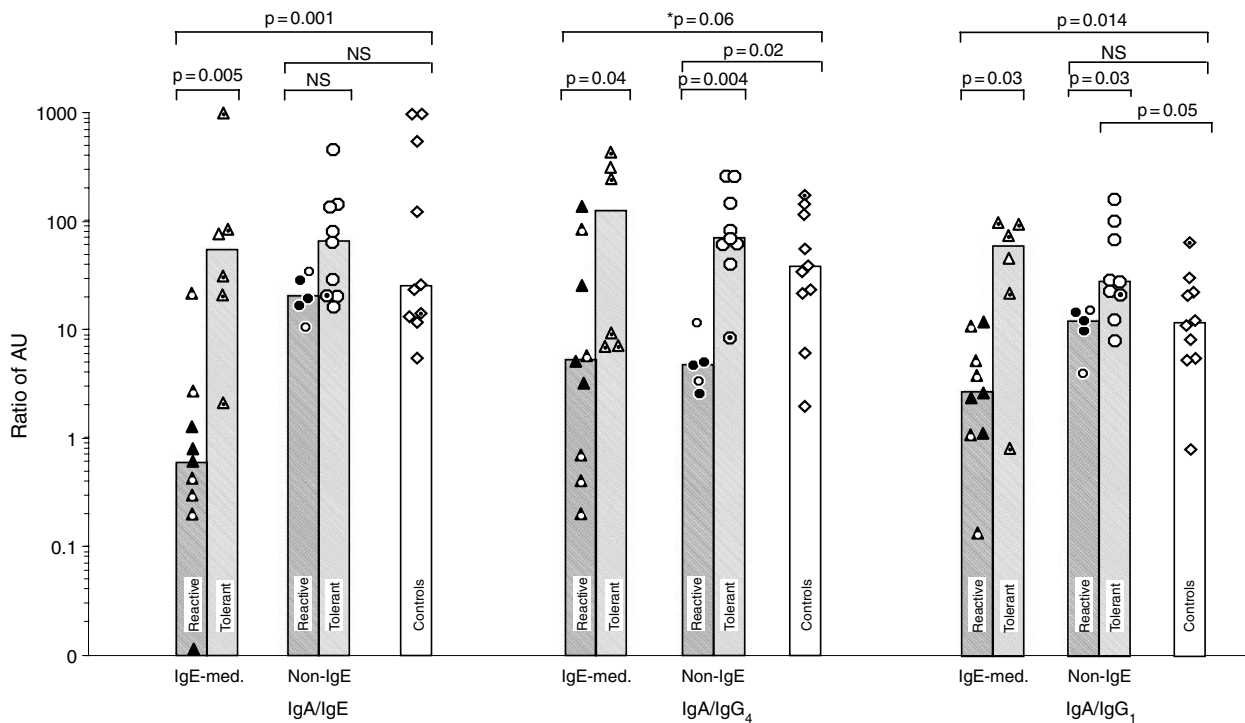


Fig. 3. Ratios of β-lactoglobulin-specific Igs in IgE- and non-IgE-mediated CMA patients: IgA/IgE, IgA/IgG₄ and IgA/IgG₁ (shown using a logarithmic scale). All three ratios were higher in tolerated patients in both patient groups. There were no significant differences between tolerant patients and controls, in either patient group. Medians are indicated by bars. Reactive patients are represented by closed symbols; tolerant patients by open symbols. Patients with other allergies than cow's milk are marked with a dot within the symbol. Ratios were compared between patient group and controls using the Wilcoxon rank sum test. $p < 0.05$ was considered significant and *p signified approaching significance.

predominantly characterized by IgG₄ and not IgE. This may explain why tolerated non-IgE-mediated patients also had significantly reduced β-LG-specific IgE levels, even though they were not elevated in clinically reactive patients.

These results are partly in disagreement with Shek et al. who reported higher β-LG-specific IgG₄ levels in CMA with allergic eosinophilic gastroenteritis, but almost no β-LG-specific IgG₄ in CMA patients with milk-sensitive enterocolitis (4). Although both patient subgroups may have been included in the present study as no gastrointestinal biopsy was taken (ethical constraint), the majority of the non-IgE-mediated CMA patients presumably had milk-sensitive enterocolitis. Methodological differences may partly explain some of this disparity as Shek et al. used 10–20 times less antigen in the ELISA coat, making it more prone to antibody interference. The only other similar study reported higher β-LG-specific IgG₄ in CMA children (<18 months) with milk-induced enterocolitis, which decreased in tolerant children, similar to the present study (22).

It has been suggested that tolerance is a consequence of an increased protective IgA

antibody response. However, this hypothesis is apparently contradicted by the higher β-LG-specific IgA levels in clinically reactive CMA, than in tolerant patients (this study) or controls (10, 23). Indeed, several earlier studies have reported similar CMP-specific IgA serum levels in CMA children (< 4 yr) and controls (17, 24, 25). The IgA system, however, may not be fully matured at this age (16), making comparison with the older children in the present study difficult. However, specific IgA serum levels may not fully reflect what happens at the gastrointestinal mucosal surfaces and it may rather be changes in the IgA ratios that are functionally important (see later).

If β-LG-specific IgG₁ may reflect an overall Th₁-dominated response, IgG₄ and IgE would reflect a Th₂-dominated, and IgA a Th3-dominated type of immune response [for references see (26)]. Although tolerization was associated with generally down-regulated β-LG-specific Ig levels, it was more pronounced for the Th₂ type (IgE, IgG₄) than for the Th₁ type of Ig (IgG₁) for both patient groups. The five-fold decreased IgG₄/IgG₁ and IgG₄/IgE median ratios in tolerated non-IgE-mediated CMA patients reflected,

therefore, predominantly an inhibited Th₂ type of immune response, which was even more pronounced for IgG₄, than for IgE, as the IgG₄/IgE median ratio decreased five-fold in tolerant patients, which in consequence induced a shift from a Th₂-, to a more normalized Th₁-dominated immune response (27). Moreover, the median 15-fold increased IgA/IgG₄ ratio suggested that the humoral immune system *de facto* shifted from an IgG₄-dominated, IgE-influenced Th₂-type, to an IgA-dominated Th₃-type of immune response after tolerization.

A similar phenomenon was observed in the IgE-mediated CMA patients. Although tolerance was associated with a nine-fold decrease in β -LG-specific IgG₁, IgE was 44-fold lower in tolerized patients. This difference between reactive and tolerant CMA patient groups has also been observed in individual patients followed in longitudinal studies (12). Surprisingly, Hill et al. were unable to observe decreased CMP-specific IgE, or any other Ig levels, during tolerance development in two longitudinal studies (3, 25). However, persistent CMA patients had higher initial levels than those who became tolerant (3), also observed in the later and more extensive study of Perry et al. (11).

The tolerized IgE-mediated CMA patients had 90-fold higher IgA/IgE, 25-fold higher IgA/IgG₄ and 23-fold higher IgA/IgG₁ median ratios because all β -LG-specific Ig levels, except IgA, were lower in the tolerized compared with the clinically reactive patients. Thus, tolerance was associated predominantly with a shift from IgE to IgA dominance. This supported the concept that at least β -LG-specific IgA may protect against allergic reactions, despite unchanged specific IgA levels and the existence of specific IgE in tolerant patients (28), because it is the IgA/IgE ratio that is functionally relevant. This concept is supported by the suppressed Th₁ and Th₂ cytokine responses observed in tolerized CMA patients (29), accompanied by increased IL-10 secretion, presumably reflecting regulatory T-cell function (Th₃ type of immune response) which stimulates specific IgA production (30). IL-10 has, moreover, a counter-regulatory role by suppressing IgE production at the same time as it increases IgG₄ production.

In contrast to non-IgE-mediated CMA patients, β -LG-specific IgG₄/IgG₁ and IgE/IgG₁ ratios in IgE-mediated CMA decreased less dramatically, explained by the similar decrease of all three Igs in tolerized patients. These results are in accord with previous studies reporting decreased IgE/IgG and IgE/IgG₁ ratios in tolerant CMA patients (12, 31).

In conclusion, whereas the changes in β -LG-specific IgE serum levels revealed its functional role in IgE-mediated CMA patients, IgG₄, or its underlying immunoregulatory mechanisms, appeared to fulfil a similar role in the non-IgE mediated CMA patients with delayed gastrointestinal symptoms. Tolerance in IgE-mediated CMA patients was associated with a dramatic increase in both IgA/IgE and IgA/IgG₄ ratios, whereas non-IgE-mediated CMA patients had an increased IgA/IgG₄ ratio only. Although both CMA patient groups may be associated with a Th₂-type of immune response, non-IgE-mediated CMA may be linked to cellular immune responses characterized by IgG₄-inducing cytokines.

References

1. SAMPSON HA. Update on food allergy. *J Allergy Clin Immunol* 2004; 113: 805–19.
2. IACONO G, CAVATAIO F, MONTALTO G, SORESI M, NOTARBARTOLO A, CARROCCIO A. Persistent cow's milk protein intolerance in infants: the changing faces of the same disease. *Clin Exp Allergy* 1998; 28: 817–23.
3. HILL DJ, FIRER MA, BALL G, HOSKING CS. Natural history of cows' milk allergy in children: immunological outcome over 2 years. *Clin Exp Allergy* 1993; 23: 124–31.
4. SHEK LP, BARDINA L, CASTRO R, SAMPSON HA, BEYER K. Humoral and cellular responses to cow milk proteins in patients with milk-induced IgE-mediated and non-IgE-mediated disorders. *Allergy* 2005; 60: 912–9.
5. SICHERER SH, EIGENMANN PA, SAMPSON HA. Clinical features of food protein-induced enterocolitis syndrome. *J Pediatr* 1998; 133: 214–9.
6. WAL JM. Bovine milk allergenicity. *Ann Allergy Asthma Immunol* 2004; 93: S2–11.
7. HUSBY S, HOST A, TEISNER B, SVEHAG SE. Infants and children with cow milk allergy/intolerance. Investigation of the uptake of cow milk protein and activation of the complement system. *Allergy* 1990; 45: 547–51.
8. HOST A, HUSBY S, GJESING B, LARSEN JN, LOWENSTEIN H. Prospective estimation of IgG, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. Levels of antibodies to whole milk protein, BLG and ovalbumin in relation to repeated milk challenge and clinical course of cow milk allergy. *Allergy* 1992; 47: 218–29.
9. SICHERER SH, SAMPSON HA. Cow's milk protein-specific IgE concentrations in two age groups of milk-allergic children and in children achieving clinical tolerance. *Clin Exp Allergy* 1999; 29: 507–12.
10. HIDVEGI E, CSERHATI E, KEREKI E, SAVILAHTI E, ARATO A. Serum immunoglobulin E, IgA, and IgG antibodies to different cow's milk proteins in children with cow's milk allergy: association with prognosis and clinical manifestations. *Pediatr Allergy Immunol* 2002; 13: 255–61.
11. PERRY TT, MATSUI EC, KAY CONOVER-WALKER M, WOOD RA. The relationship of allergen-specific IgE levels and oral food challenge outcome. *J Allergy Clin Immunol* 2004; 114: 144–9.

12. JAMES JM, SAMPSON HA. Immunologic changes associated with the development of tolerance in children with cow milk allergy. *J Pediatr* 1992; 121: 371–7.
13. BEYER K, TEUBER SS. Food allergy diagnostics: scientific and unproven procedures. *Curr Opin Allergy Clin Immunol* 2005; 5: 261–6.
14. AALBERSE RC, SCHUURMAN J. IgG4 breaking the rules. *Immunology* 2002; 105: 9–19.
15. VERCELLI D. One cytokine, two isotypes: a trojan horse, pandora's box, and an evolving paradigm. *Am J Respir Crit Care Med* 2000; 162: S86–90.
16. MAYER L. Mucosal immunity. *Pediatrics* 2003; 111: 1595–600.
17. ISOLAURI E, SUOMALAINEN H, KAILA M, et al. Local immune response in patients with cow milk allergy: follow-up of patients retaining allergy or becoming tolerant. *J Pediatr* 1992; 120: 9–15.
18. HOST A. Frequency of cow's milk allergy in childhood. *Ann Allergy Asthma Immunol* 2002; 89: 33–7.
19. SAMPSON HA, ANDERSON JA. Summary and recommendations: classification of gastrointestinal manifestations due to immunologic reactions to foods in infants and young children. *J Pediatr Gastroenterol Nutr* 2000; 30(suppl.1) S87–94.
20. HVATUM M, SCOTT H, BRANDTZAEG P. Pitfalls in determining IgG and IgG subclass antibodies to food antigens. *J Immunol Methods* 1992; 148: 77–85.
21. EL RAFAI A, PETERS SM, HARRIS N, BELLANTI JA. Diagnostic value of IgG4 measurements in patients with food allergy. *Ann Allergy* 1989; 62: 94–9.
22. BURKS AW, WILLIAMS LW, CASTEEL HB, FIEDOREK SC, CONNAUGHTON CA. Antibody response to milk proteins in patients with milk-protein intolerance documented by challenge. *J Allergy Clin Immunol* 1990; 85: 921–7.
23. KOKKONEN J, TIKKANEN S, KARTTUNEN TJ, SAVILAHTI E. A similar high level of immunoglobulin A and immunoglobulin G class milk antibodies and increment of local lymphoid tissue on the duodenal mucosa in subjects with cow's milk allergy and recurrent abdominal pains. *Pediatr Allergy Immunol* 2002; 13: 129–36.
24. FIRER MA, HOSKINGS CS, HILL DJ. Humoral immune response to cow's milk in children with cow's milk allergy. Relationship to the time of clinical response to cow's milk challenge. *Int Arch Allergy Appl Immunol* 1987; 84: 173–7.
25. HILL DJ, FIRER MA, BALL G, HOSKING CS. Recovery from milk allergy in early childhood: antibody studies. *J Pediatr* 1989; 114: 761–6.
26. HALSTENSEN TS. Why are we not all allergic: basic mechanisms for tolerance development. *Environ Toxicol Pharmacol* 1997; 4: 25–31.
27. SCHADE RP, VAN IEPEREN-VAN DIJK AG, VAN REUSEN FC, et al. Differences in antigen-specific T-cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of TH2 cytokines. *J Allergy Clin Immunol* 2000; 106: 1155–62.
28. ISOLAURI E, VIRTANEN E, JALONEN T, ARVILOMMI H. Local immune response measured in blood lymphocytes reflects the clinical reactivity of children with cow's milk allergy. *Pediatr Res* 1990; 28: 582–6.
29. TIEMESSEN MM, VAN IEPEREN-VAN DIJK AG, BRUIJNZEEL-KOOMEN CA, GARSSEN J, KNOL EF, VAN HOFFEN E. Cow's milk-specific T-cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. *J Allergy Clin Immunol* 2004; 113: 932–9.
30. HORWITZ DA, ZHENG SG, GRAY JD. The role of the combination of IL-2 and TGF-beta or IL-10 in the generation and function of CD4+ CD25+ and CD8+ regulatory T cell subsets. *J Leukoc Biol* 2003; 74: 471–8.
31. DANNAEUS A, INGANAS M. A follow-up study of children with food allergy. Clinical course in relation to serum IgE- and IgG-antibody levels to milk, egg and fish. *Clin Allergy* 1981; 11: 533–9.