

Casein-specific immunoglobulins in cow's milk allergic patient subgroups reveal a shift to IgA dominance in tolerant patients

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Differences in casein-specific immunoglobulin (Ig) G-subclass and IgA serum levels between reactive and tolerant patients may hint at the immunopathogenesis during tolerance development in cow's milk allergy (CMA). α -, β - and κ -casein-specific IgG₁, IgG₄, IgE and IgA serum levels were compared in clinically reactive and tolerized IgE-mediated (n = 15) and non-IgE-mediated (n = 14) CMA with delayed gastrointestinal symptoms, using enzyme-linked immunosorbent assay (ELISA) and immunoblot techniques. The median anti-casein IgE levels in clinically reactive IgE-mediated CMA patients (n = 9) were 140- to 180-fold higher than in tolerized patients (n = 6) and 160- to 200-fold higher than in controls (n = 10). Median α -, β - and κ -casein-specific IgG₁ and IgG₄ levels were nine- to 60-fold higher in reactive patients and five- to 60-fold in tolerized patients. Clinical tolerance in IgE-mediated CMA was thus associated with decreased casein-specific IgE, IgG₄ and IgG₁, whereas serum IgA anti- α -, β - and κ -casein remained practically unaltered. Tolerized cow's milk protein (CMP)-sensitive atopic dermatitis had, in particular, decreased κ -casein-specific IgG₁ levels, compared with clinically reactive patients. The ELISA levels to immunoblot correlation profile for the α -, β - and κ -casein-specific IgE suggested that the IgE-mediated CMA patients predominantly reacted to tertiary α - and β -casein epitopes whereas the IgE in non-IgE-mediated patients reacted to linearized α -, β - and κ -casein epitopes. Clinical tolerance in non-IgE-mediated CMA patients (n = 9) was associated with a four- to 10-fold decrease in casein-specific IgE levels, accompanied by a five- to eightfold decrease in IgG₁ and five- to 60-fold decrease in IgG₄ levels, whereas casein-specific IgA levels remained unaltered. Thus, tolerance in both patient groups was characterized by a generalized decreased humoral immune response to caseins, which induced a functional shift to IgA dominance.

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Casein is one of the major cow's milk allergens in immunoglobulin (Ig) E-mediated cow's milk allergy (CMA) (1, 2). Allergic reactions to casein appear to have become more important during the last decades (3). Casein consists of α -, β - and κ -caseins and most IgE-mediated CMA patients have similarly elevated IgE and IgG levels to more

than one casein (1, 4, 5), but not necessarily to the same fraction (6). A similar polyisotypic response to α -, β - and κ -caseins has been reported in non-IgE-mediated CMA patients (7). Although the casein-specific IgG in IgE-mediated CMA appears to have a minor immunopathological role, it may be involved in non-IgE-mediated delayed CMA.

IgG consists of four subclasses with different immunopathological properties. Whereas IgG₁ is related to inflammatory Th₁-driven immune regulation (8), IgG₄ appears to be associated with IgE in a Th₂ immune regulatory fashion [reviewed in (9)]. The previously described increased casein-specific IgG levels in IgE-mediated CMA patients (1, 5) may therefore have included both IgG₄ as well as IgG₁ (10). Tolerance has been suggested to be associated with increasing cow's milk protein (CMP)-specific IgA (11) because it may inhibit IgG/IgE-mediated inflammatory pathways (12). However, this has been difficult to confirm and tolerized CMA patients have both unchanged (13, 14) and decreased CMP-specific serum IgA levels (15, 16).

Therefore, the aim of this study was to examine the humoral immune responses to casein subfractions in clinically reactive and tolerized CMA patients, with both IgE-mediated CMA and non-IgE-mediated CMA with delayed gastrointestinal reactions. α -, β - and κ -casein-specific IgG, IgG₁, IgG₄, IgE and IgA levels were compared using enzyme-linked immunosorbent assay (ELISA) and, for IgE, also immunoblots. Antigen-specific immunoglobulin ratios were calculated to better illustrate the immunoregulatory events, which may be associated with tolerance development.

Materials and methods

Study population

Patients with CMA who were selected for the study had been referred by primary practitioners and/or hospital clinics to the specialist clinic at BKL Voksentoppen, National Hospital (Oslo, Norway, (Table 1). These patients had a previous history of clinically controlled milk challenge-induced reactions while on a milk-free diet. The 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. All the children, except for four highly reactive IgE-mediated CMA children ($n = 27$, mean age 7.4 yr, range 4–13 yr; 10 female and 17 male), were retested under supervision of the same clinician (RH), in a 3-day open clinical challenge, followed up to 1 month at home.

Open food challenge was performed with semi-skimmed milk in pancakes for the first 2 days, according to the sensitivity of the patients. Highly sensitive patients were given 10 ml the first day and 100 ml the next day, both in three doses (Table 1). If the milk was

tolerated, then half to one portion of yoghurt (175 ml) or one to two slices of cheese were given on the third day. Less sensitive patients were started with 100 ml milk on the first day (in three doses) and yoghurt/cheese on the next 2 days. The age of the patients was taken into account when deciding the amount of cheese or yoghurt.

Immediate symptoms included itching, urticaria, eczema, asthma, vomiting, abdominal pain, diarrhoea or anaphylaxis. Patients with atopic dermatitis were confirmed challenge positive when milk intake resulted in an immediate exacerbation of the cutaneous symptoms (17). Delayed symptoms, such as abdominal pain, loose stools, diarrhoea or delayed eczematous reactions occurred from 24 h, up to 7–10 days following the start of the challenge period. Two additional tolerant patients (male, 21 and 23 yr), with a clinical history of CMA and who were 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 ($n = 23$) or consumed milk products ($n = 6$).

Grouping patients by symptoms or symptom onset time alone may not discriminate underlying mechanisms, as discussed elsewhere (18). Patients were therefore divided into two groups, on the basis of symptoms, onset time and CMP-specific IgE levels (≤ 3.5 kU/l). Patients with a clinical history of IgE-mediated CMA with CMP-specific IgE levels ≤ 3.5 kU/l, with or without milk-exacerbated atopic dermatitis ($n = 15$, five female, 10 male; mean age 9.7 yr, range 4–23 yr) were separated from patients with milk-induced delayed gastrointestinal reactions ($n = 14$, five female, nine male; mean age 7.1 yr, range 4–11 yr). Ten of the patients with IgE-mediated CMA and three of the non-IgE-mediated CMA patients also had reactions to other foods, most frequently to eggs and fish. The control group ($n = 10$, mean age 37.1 yr, range 1–64 yr), consisted of two non-allergic, milk-consuming children and eight adults (six female and four male). One control subject was mildly allergic to wheat.

Blood samples were obtained prior to challenge. Cow's milk-specific IgE serum levels were measured using the UniCAP-Specific IgE FEIA system (Pharmacia Diagnostics AB, Uppsala, Sweden), with skimmed milk as antigen. The mean milk-specific IgE level in clinically reactive IgE-mediated CMA patients (7/9) was 64 kU/l (range 10 to > 100 kU/l). Two clinically reactive non-IgE-mediated CMA patients had 2 kU/l milk-specific IgE. All other patients and all control subjects had < 0.35 kU/l milk-specific

Table 1. Clinical features of individual patients

Patient	Age (yr)	Gender	Milk tolerance	OFC dose for reaction (ml)	Reaction	Reactions to other food	Total IgE (kU/l)	Specific IgE (kU/l)	SPT
Group I: IgE-mediated CMA, with/without atopic dermatitis (n = 15)									
MA1	4.4	F	+	—	—	—	598	<0.35	Negative
MA8	12.8	F	—	100	Itching	Egg, fruit	169	<0.35	Negative
MA9	12.4	F	—	100	Itching, diarrhoea	Egg, fish	3342	<0.35	Negative
MA10	8.5	M	—	100	Itching	Egg, fish	1500	59.4	+++(+)
MA13	23.2	M	+	—	—	Fruit/nuts, pollen	183	<0.35	n.a.
MA14	21.8	M	+	—	—	Fruit/nuts, pollen	9.36	<0.35	n.a.
MA21	13.2	M	+	—	—	Egg, fish	707	0.56	++(+)
MA22	4.0	F	—	100	Atopic dermatitis	Fish	81	10.4	+++(+)
MA23	7.3	M	—	100	Itching, atopic dermatitis	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	—	100	Diarrhoea	—	<63	<0.35	Negative
MA4	8.2	M	—	100	Diarrhoea	—	<63	<0.35	Negative
MA5	6.4	F	+	—	—	—	6	<0.35	Negative
MA7	7.0	M	—	100	Pain, diarrhoea	Fish, egg	571	2.0	++
MA11	6.7	M	+	—	—	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	—	100	Diarrhoea	—	<70	<0.35	Negative
MA29	10.0	M	—	100	Pain, diarrhoea	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 to 2)									
Group III: healthy controls (n = 10)									
NC5	4.0	M	+	n.a.	—	—	353	<0.35	Negative
NC6	58	M	+	n.a.	—	—	544	<0.35	n.a.
MA20	0.9	F	+	n.a.	—	—	<2.0	<0.35	Negative
NC7	22	F	+	n.a.	—	—	12.8	<0.35	n.a.
NC8	6	F	+	n.a.	—	—	134	<0.35	n.a.
NC9	53	F	+	n.a.	—	—	2.22	<0.35	n.a.
NC10	60	F	+	n.a.	—	—	6.6	<0.35	n.a.
NC11	37	M	+	n.a.	—	—	<2.0	<0.35	n.a.
NC12	31	M	+	n.a.	—	—	7.9	<0.35	n.a.
NC13	39	F	+	n.a.	—	Wheat, pollen	746	<0.35	n.a.

Patients were recruited to the study and grouped on the basis of symptoms, onset time and CMP-specific IgE-levels at the time of initial diagnosis: (a) patients with CMP-specific IgE-levels ≤ 3.5 kU/l and a clinical history of IgE-mediated cow's milk allergy (CMA), including milk-exacerbated atopic dermatitis and (b) patients with milk-induced delayed gastrointestinal reactions. The table shows results from subsequent retesting, including open food challenge (OFC) dose and response, total and CMP-specific IgE and skin-prick test (SPT). SPT reactions: wheal equal to that from positive control (histamine chloride, 10 mg/ml), +++; wheal half of the histamine wheal, ++; wheal double the histamine wheal, ++++; n.a., not applied; *, not performed due to risk of severe reaction.

IgE. Skin-prick tests (SPT) were performed on the volar side of the forearm with a lancet with 1-mm tip and shoulder (ALK-Abellø, Copenhagen, Denmark) and allergenic extract of cow's milk (ALK-Abellø). The reaction was read after 15 min and the wheal was measured and compared with the wheal elicited by histamine chloride (10 mg/ml). A wheal equal to that of histamine was denoted + + +, half of the

histamine wheal + + and double the histamine wheal + + + +. Clinically reactive IgE-mediated CMA patients (7/9) had responses ranging from + + + (n = 4) to + + + + (+) and two tolerated patients in the same group had responses of + + and + + (+) respectively. Two clinically reactive non-IgE-mediated CMA patients had SPT responses of + +. All other subjects had negative SPT.

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. The inclusion of age-matched non-allergic control subjects, although optimal, was not possible for ethical reasons, and the control group consisted mainly of adult subjects.

ELISA for α -, β - and κ -casein-specific Ig

The purity of the antigens was confirmed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as described below. The ELISA procedure has been detailed previously (18). Briefly, plates were coated overnight at 4°C, with α - or β -casein (1 μ g/ml) or κ -casein (10 μ g/ml) (all Sigma-Aldrich, Steinheim, Germany) in carbonate/bicarbonate buffer (pH 9.6), and blocked with phosphate-buffered saline containing 1% human serum albumin (Calbiochem, La Jolla, CA, USA), at 37°C for 2 h. A standard curve was prepared from a serially diluted high CMP-specific IgE titre serum pool (21 patients). Standard, suitably diluted patient samples or buffer blanks (duplicates) were incubated (37°C, 1 h) in a humid atmosphere. 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 Dako Cytomation, Glostrup, Denmark), followed by goat-anti-rabbit IgG-horse radish peroxidase (HRP) (Zymed) or rabbit-anti-mouse IgG-HRP (DakoCytomation), were added, as appropriate. Enhanced K-Blue TMB substrate (Neogen, Lexington, KY, USA) was then added 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

Purified, lipopolysaccharide-depleted α -, β - and κ -caseins (1 μ g of each/lane; Sigma-Aldrich), were separated at 200 V on 12% polyacrylamide gels, using the Mini-Protean III (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The proteins were electrophoretically transferred (70 V, 1 h, RT) on to Protran BA83 0.2 μ m nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).

Tris-buffered saline/0.3% Tween/3% gelatine (Sigma-Aldrich) was used as blocking and assay buffer. The purity of the α -, β - and κ -casein subfractions was confirmed using chicken polyclonal anti- α -, β - and κ -casein antisera (Ag-Research, Ruakura, NZ, USA) and rabbit-anti-chicken IgY-HRP conjugate (Sigma-Aldrich). After blocking for 1 h, membrane strips were incubated overnight at 4°C with patient sera (1/10). A serum with no detectable CMP-specific IgE (CAP < 0.35 kU/l) was used as negative control and a positive IgE control was obtained by adding human IgE myeloma protein at 250 ng/ml to this serum. Blots were then incubated for 1.5 h in rabbit-anti-human IgE (Dako), followed by goat-anti-rabbit IgG-HRP conjugate, which was developed with TMB (both Zymed), and scanned (HP Scanjet, Hewlett-Packard, CO, USA). Band intensities were measured as pixel volumes, using TotalLab analytical software (Nonlinear Dynamics Ltd, Newcastle, UK).

Statistical analysis

Immunoglobulin values were not normally distributed (Shapiro-Wilk *W* test) so nonparametric tests were applied. Ig values and ratios measured using ELISA, and immunoblot pixel volumes 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

Antigen-specific Ig levels measured by ELISA will be dependent on the sum of the total affinities and avidities of the Ig molecules. Ratios of the α -, β - and κ -casein-specific Ig levels give an indication of overall immune response and type of regulatory events associated with tolerance. It has been suggested that high titres of antigen-specific IgA may have a blocking effect on IgG or its subclasses, but the relative distribution of the subclasses has been shown to be relatively unaffected by the removal of IgA (19). IgA was therefore not removed from the samples.

Any major contribution due to unexpectedly high levels of α -, β - and κ -casein-specific IgG₂ and IgG₃ was excluded by comparing α -, β - and κ -casein-specific pan-IgG levels with the combined α -, β - and κ -casein-specific IgG₁ and IgG₄ levels. All pan-IgG levels were found to be highly correlated with the corresponding combined IgG₁ and IgG₄ levels in IgE-mediated CMA

patients and non-IgE-mediated CMA patients (both $p < 0.0001$) and controls ($p = 0.0005$).

Casein in CMP consists predominantly of α -, β - and κ -casein, whose individual allergenicities have been established previously (4). Combining α -, β - and κ -casein-specific Ig values in AU/ml, to give an estimated total casein reactivity index, thus enables comparison with other studies in the literature where only total casein has been estimated. However, it is noted that the possibility of cross-reactivity to common casein may produce a higher estimate of total casein reactivity than if whole casein binding was measured in a separate ELISA. However, this would not affect comparisons between patient groups.

Results

Patients

Four of the patients who were considered to be clinically reactive had very high CMP-specific IgE levels (> 47 kU/l), strong CMP-specific SPT (3+) and a clinical history of severe cow's milk reactions. The risk of severe reaction was therefore considered too high to justify cow's milk challenge (MA26, 27, 28 and 31). The remaining 25 patients underwent a clinically controlled open cow's milk challenge test, which revealed that 15 were clinically tolerant, six in the IgE-mediated CMA group and nine in the non-IgE-mediated CMA group. The dose of skimmed milk eliciting symptoms in the 14 reactive patients was 100 ml.

Higher casein-specific IgE, IgG₁ and IgG₄ levels in clinically reactive IgE-mediated CMA patients

Reactive patients had 140- to 180-fold higher α -, β - and κ -casein-specific IgE levels compared with tolerized patients ($p < 0.05$), and 160- to 200-fold higher than controls ($p < 0.005$, Fig. 2a–c). Moreover, reactive patients had five- to eightfold higher α -, β - and κ -casein-specific IgG₁ levels than tolerized patients ($p < 0.05$), and nine- to 13-fold higher than controls ($p < 0.05$). Reactive patients also had 38- to 60-fold higher median α -, β - and κ -casein-specific IgG₄ levels than controls ($p < 0.01$). However, although reactive patients had five- to 60-fold higher median α -, β - and κ -casein-specific IgG₄ levels than tolerized patients, this was not significant due to overlapping values (Fig. 2a–c). Combining the Ig reactivity to the casein fractions revealed that clinically reactive IgE-mediated CMA patients had 160-fold higher median estimated total casein-specific IgE, 10-fold higher IgG₁ and 11-fold higher IgG₄ reactivity compared with tolerized patients ($p < 0.05$, Fig. 1).

Non-IgE-mediated CMA patients

Although the clinically reactive non-IgE-mediated patients with delayed gastrointestinal reactions had significantly higher β - (IgE and IgG₄) and κ -casein (IgE) Ig titres than tolerized patients, it was the α -casein reactivity that dominated (Fig. 2a–c). The clinically reactive patients had eightfold higher median IgG₁

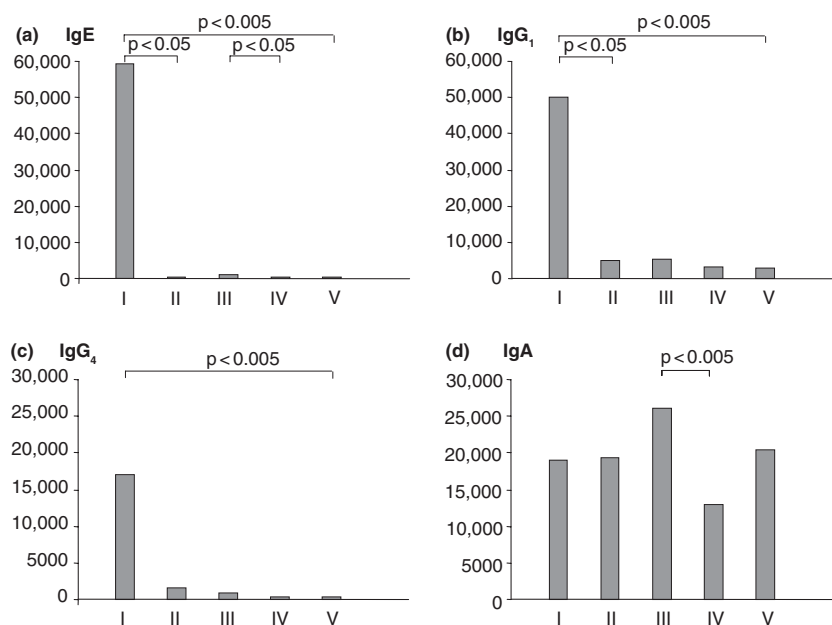
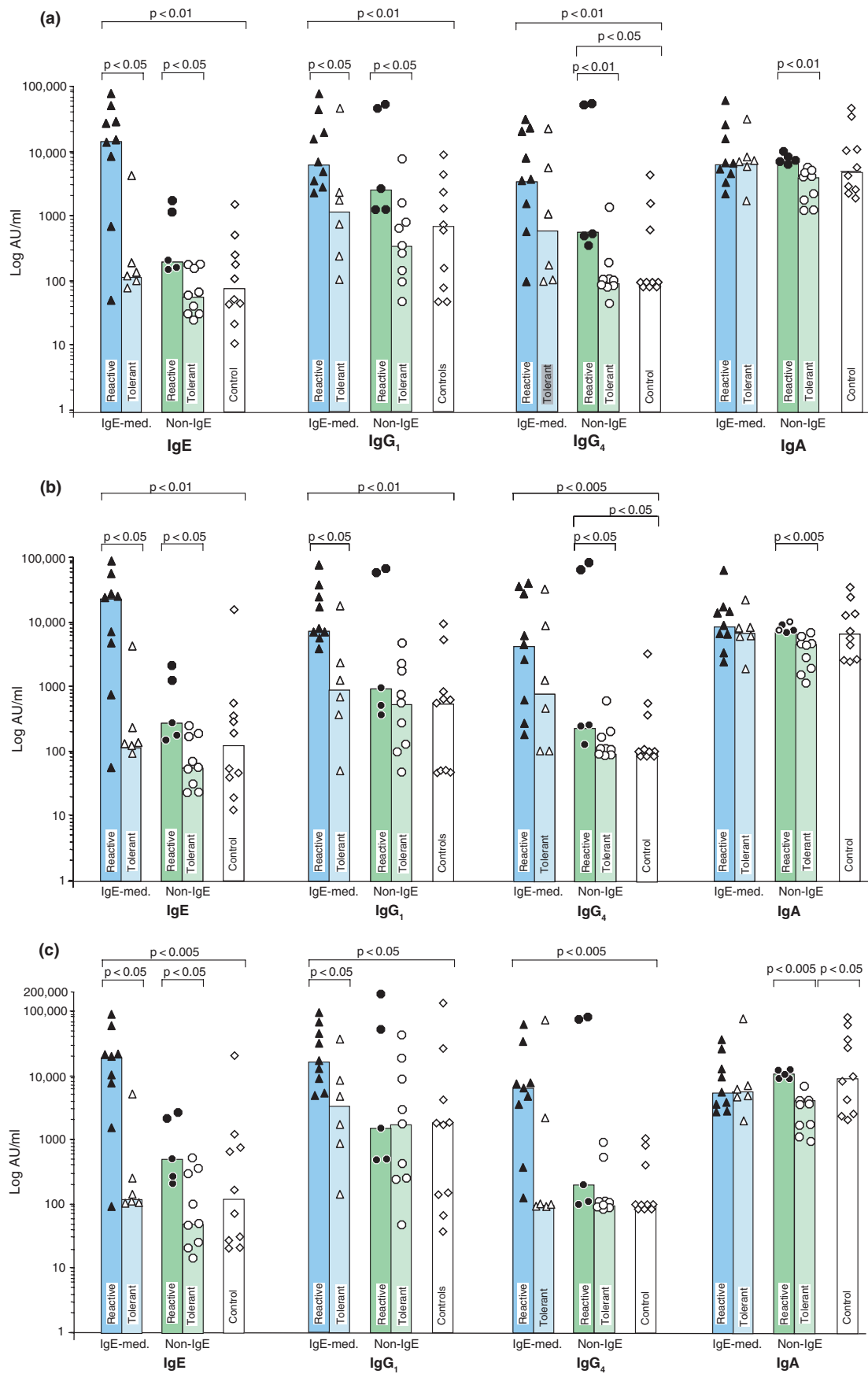


Fig. 1. Estimated median total casein-specific immunoglobulin (Ig) in AU/ml. I, clinically reactive IgE-mediated cow's milk allergy (CMA); II, tolerized IgE-mediated CMA; III, reactive non-IgE-mediated CMA; IV, tolerized non-IgE-mediated CMA; V, healthy controls.



($p < 0.01$), sixfold higher IgG₄ ($p < 0.05$) and 3.5-fold higher α -casein-specific IgE levels ($p < 0.05$, Fig. 2a–c).

α -, β - and κ -casein-specific Ig ratios revealed no significant differences between reactive and tolerized patients

Combining α -, β - or κ -casein-specific Ig values to give IgG₄/IgG₁, IgG₄/IgE and IgE/IgG₁ ratios revealed no significant differences between clinically reactive patients with IgE- or non-IgE-mediated CMA, when compared with either tolerized patients or controls, presumably because the casein-specific IgG₁, IgG₄ and IgE levels were all similarly decreased in tolerized patients (data not shown).

Tolerized IgE-mediated CMA patients had increased casein-specific IgA/IgE ratios

The tolerized IgE-mediated CMA patients had 43- to 71-fold increased α -, β - and κ -casein-specific IgA/IgE ratios compared with reactive patients ($p < 0.05$, Fig. 3), mainly because tolerized patients had markedly lower casein-specific IgE levels.

Although a similar trend was noted for non-IgE-mediated CMA patients (two- to threefold

increased α -, β - and κ -casein-specific IgA/IgE ratios in tolerized patients), it did not reach statistical significance (Fig. 3).

Similarly, the median two- to threefold increased α -casein-specific IgA/IgG₁ and IgA/IgG₄ ratios, observed in tolerized non-IgE-mediated CMA patients, did not reach significance due to the decrease in both α -casein-specific IgA, IgG₁ and IgG₄ levels. This was even more pronounced in β -casein, for which the IgA/IgG₁ and IgA/IgG₄ ratios were unchanged. Median κ -casein-specific IgA/IgG₁ and IgA/IgG₄ ratios both in fact decreased fourfold, reflecting the greater decrease in κ -casein-IgA levels, rather than changes in IgG₁ or IgG₄ (Fig. 3).

Only tolerized non-IgE-mediated CMA patients had decreased casein-specific IgA levels compared with reactive patients and controls

Clinically reactive non-IgE-mediated patients had significantly two- to threefold higher median α -, β - and κ -casein-specific IgA levels than tolerized patients ($p < 0.005$). The κ -casein-specific IgA levels were, in particular, decreased in tolerized patients compared with controls ($p < 0.05$, Fig. 2a–c). In contrast, clinically

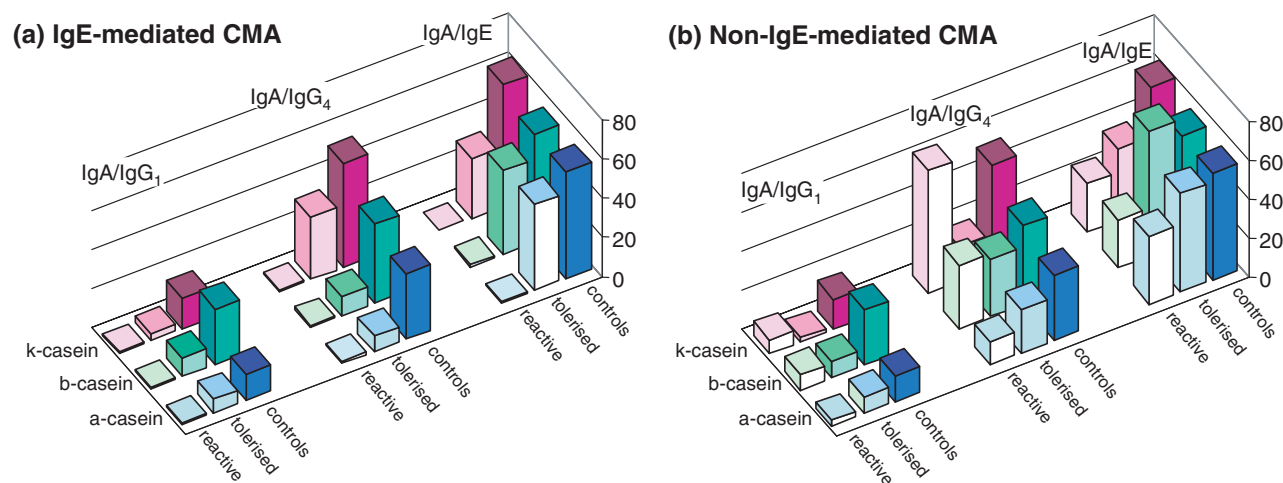


Fig. 3. Median ratios of α -, β - and κ -casein-specific immunoglobulins (Ig) in (a) IgE- and (b) non-IgE-mediated cow's milk allergy (CMA) patients: IgA/IgG₁, IgA/IgG₄ and IgA/IgE. All three ratios showed an increasing trend from clinically reactive (lightly shaded) to tolerized IgE-mediated CMA patients (shaded green), to controls (heavily shaded). In non-IgE-mediated CMA patients, only α -casein ratios (shaded blue) showed a similar trend; β -casein ratios (shaded green) increased or were unchanged and κ -casein IgA/IgG₁ and IgA/IgG₄ ratios (shaded red) were greater in reactive than in tolerized patients, because of the decrease in IgA levels.

Fig. 2. (a) α -Casein-specific, (b) β -Casein-specific, and (c) κ -Casein-specific IgE, IgG₁, IgG₄ and IgA levels in AU/ml (shown using a logarithmic scale) measured with ELISA in IgE-mediated (shaded blue) and non-IgE-mediated CMA patients (shaded green). Median values are indicated by bars. Reactive patients are represented by closed symbols; tolerant patients by open symbols. Ig values were compared between patient groups and controls using the Wilcoxon rank sum test.

reactive and tolerized IgE-mediated CMA patients and controls had similar α -, β - and κ -casein-specific IgA levels (Figs 1 and 2a–c).

α -, β - and κ -casein-specific IgE detected by immunoblotting

The clinically reactive IgE-mediated CMA patients had significantly ($p < 0.05$) higher median IgE pixel volumes to α -casein (49,994), β -casein (21,795) and κ -casein (34,800) compared with tolerized patients (9748, 4745, 7774 respectively) and controls (3551, 3124, 164, respectively, $p < 0.05$; not shown). Surprisingly, only the immunoblot-detected κ -casein-specific IgE correlated significantly to the ELISA-detected κ -casein-specific IgE level ($p < 0.005$, $R^2 = 0.5558$) in the IgE-mediated CMA patients.

The non-IgE-mediated patients had similar casein-specific IgE pixel volumes to controls. Clinically reactive non-IgE-mediated patients had a significantly higher pixel volume for κ -casein only ($p < 0.05$), compared with tolerized patients. Interestingly, the immunoblot-detected casein-specific IgE pixel volumes in these non-IgE-mediated patients correlated highly significantly with the ELISA-detected levels for both α -casein ($p < 0.0001$, $R^2 = 0.8651$), β -casein ($p < 0.0001$, $R^2 = 0.8035$) and κ -casein ($p < 0.0005$, $R^2 = 0.6916$).

Discussion

The present study compared the α -, β - and κ -casein-specific Ig levels in clinically reactive IgE-mediated and non-IgE-associated CMA patients with patients subsequently found to be tolerant on rechallenge.

Although patients with IgE-mediated CMA have increased CMP-specific IgE serum levels, IgE may still be present in tolerized patients and controls (20). Hill et al. (14, 21) were actually unable to detect any significant decrease in CMP-specific IgE, or any other Ig levels, during tolerance development. Tolerance was therefore suggested to be due to increased CMP-specific IgA levels (11), as sIgA may inhibit allergen penetration, and serum IgA may block its binding to IgE-coated mucosal mast cells. However, the CMP-specific IgE serum levels were also reported to decrease as IgE-mediated CMA patients become tolerant (10, 22). This is in agreement with the present 140- to 180-fold decreased α -, β - and κ -casein-specific IgE levels in the tolerized IgE-mediated CMA patients. This also concurs with Perry et al. (23), who observed higher CMP-specific IgE levels in CMA patients who responded to cow's milk challenge,

compared with those who had become tolerant. The casein-specific IgE is rarely monospecific (4, 7), and most IgE-mediated CMA patients in the present study had similar IgE ELISA levels to all casein fractions, suggesting either cross-reactive casein epitopes or co-regulated immune response. The α - and β -casein-specific IgE from these patients may, moreover, recognize both conformational, tertiary structures and linear epitopes in these fractions, as the ELISA-measured α - and β -casein-specific IgE levels (detecting tertiary epitopes) did not correlate to the immunoblot-generated pixel values. This contrasted with the highly significant correlation between ELISA- and immunoblot-detected κ -casein-specific IgE values, suggesting that the dominant κ -casein epitopes were linearized. This is in agreement with linear κ -casein epitopes recognized by IgE from patients with IgE-mediated CMA (24). However, these patients also had predominantly linear IgE-binding epitopes on α s₁-casein (25), α s₂-casein (26) and β -casein (24). The authors have concluded that casein tertiary structure was lost during digestion, and assumed the survival only of linear epitopes. However, the techniques used would not identify tertiary structures, and the present study suggested that some IgE binding to more intact, conformational epitopes on α - and β -casein takes place in IgE-mediated CMA patients.

Whereas IgG₄ was previously thought to have a functional role in food allergy (27), it is now considered to be protective, as it may compete for antigen binding and has few known inflammatory functions. However, casein-specific IgG₄ responses were significantly higher in reactive IgE-mediated CMA patients, and, furthermore, slightly, although not significantly, decreased in tolerized patients. Tolerized patients had in fact a decrease in all systemic casein-specific Ig, which was reflected by the minimal changes in α -, β - or κ -casein-specific immunoglobulin ratios (IgG₄/IgG₁, IgG₄/IgE and IgE/IgG₁). This is somewhat in contrast to James and Sampson (10), who observed that although all casein-specific Ig decreased, the IgE decreased more, so the casein-specific IgE/IgG₁ and IgE/IgG₄ ratios decreased in tolerized IgE-mediated CMA patients.

While IgG₄ is regulated by an IgE-associated Th₂ type of immune response (9), IgG₁ characterizes a Th₁ type of response. The elevated casein-specific IgG₁ in the IgE-mediated CMA patients may therefore have reflected combined Th₂–Th₁ associated immune responses (Th₀), as suggested in atopic dermatitis (28). Interestingly, although the reactive IgE-mediated CMA

patients had similar IgG₁ responses to all caseins, regardless of whether they had CMP-sensitive atopic dermatitis or not, the tolerized atopic dermatitis patients had significantly lower IgG₁ immune response to κ -casein, only. This is partly in agreement with the regulatory T-cell dependent, decreased κ -casein-induced lymphocyte proliferation in these tolerized patients (29). Thus κ -casein may, in particular, be involved in CMP-sensitive atopic dermatitis. This is in agreement with Werfel *et al*, who isolated κ -casein-specific T cell clones from such patients (30), producing mainly the Th₁ cytokine, interferon- γ (IFN- γ) (28).

It has been suggested that a Th₁ cellular immune response may be involved in the pathogenesis of non-IgE-mediated CMA with gastrointestinal symptoms, because there are 10 times more IFN- γ - than interleukin-4-secreting lymphocytes in the duodenal lamina propria of cow's milk-specific enteropathy patients (31). However, the present non-IgE-mediated CMA patients showed little evidence for a Th₁-mediated immunopathology. There was no obvious IgG₁ response to any one casein fraction, although tolerized patients had significantly decreased α -casein-specific IgG₁. Clinically reactive non-IgE-mediated CMA patients had significantly increased IgG₄ levels to both α - and β - (but not κ -) caseins, which were also significantly decreased in tolerized patients, suggesting a Th₂-associated rather than a Th₁-associated immune response in this patient group. This is supported by the increased casein-specific IgG₄ in CMA children (<18 months) with milk-induced enterocolitis, and its decrease in tolerized children (32).

Tolerance has been suggested to be the consequence of an increased mucosal IgA antibody response (11). This is difficult to confirm as clinically reactive non-IgE-mediated CMA patients actually had higher casein-specific serum IgA levels than tolerized patients (this study) or controls (15, 16). Clinically reactive CMA children (<4 yr) who had similar CMP-specific serum IgA levels to controls (13, 14, 33), may not be directly compared with the current older children as they may not have had a fully matured mucosal IgA production system (12).

The functional outcome may be more dependent on antigen-specific Ig-type/subtype ratios than on absolute levels. This is supported by the 43- to 71-fold increase in median α -, β - and κ -casein-specific IgA/IgE ratios in the tolerized IgE-mediated CMA patients. Although casein-specific IgA levels were similar in reactive and tolerized patients, the decreased casein-specific

IgE and IgG levels in tolerant patients resulted in a shift to IgA dominance, supporting a protective role for IgA (33), because the IgA/IgE ratio is more functionally relevant than the IgA level in itself.

In conclusion, although the increased casein specific IgE in IgE-mediated CMA was, in particular, decreased in tolerized patients, IgG₁ and IgG₄ decreased as well, reflecting both the immunopathological role for IgE, as well as a generalized reduced humoral immune response to caseins, which did not involve IgA. The markedly reduced IgG₁ response to κ -casein in tolerized CMP-sensitive atopic dermatitis patients suggested a role for κ -casein-specific IgG₁ in the immunopathology of the atopic dermatitis. Although non-IgE-mediated CMA patients had increased casein-specific IgE, IgG₁ and IgG₄, all decreased similarly in tolerized patients. Thus, non-IgE mediated CMA with delayed gastrointestinal reactions involves both Th₁ and Th₂ regulatory events which appear to be simultaneously downregulated in tolerized patients, presumably reflecting increased regulatory T-cell function (34, 35).

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