

Original article

Humoral and cellular responses to cow milk proteins in patients with milk-induced IgE-mediated and non-IgE-mediated disorders

Background: Cow milk allergy (CMA) is one of the most common food allergies in childhood. Patients with CMA present with a wide range of immunoglobulin (Ig)E- and non-IgE-mediated clinical syndromes. Limited information is known about the specific humoral and cellular responses to cow milk proteins in these various forms of CMA.

Objective: The aim of the study was to determine IgE, IgA, IgG₁ and IgG₄ antibody levels and lymphocyte proliferative responses to the major cow milk allergens in patients with IgE- and non-IgE-mediated CMA.

Methods: One hundred and forty cow milk allergic patients, 6 months to 22 years of age, were included in the study. One hundred and thirteen patients had IgE-mediated CMA, 11 had milk protein-induced enterocolitis syndrome and 16 had allergic eosinophilic gastroenteritis. Twenty-one patients without food allergy, 8 months to 18 years of age, served as controls. Serum IgE, IgA, IgG₁ and IgG₄ antibodies to α -, β -, and κ -casein, α -lactalbumin and β -lactoglobulin were measured using enzyme-linked immunosorbent assays. For a subset of these patients, we performed lymphocyte proliferation assays to the various milk allergens.

Results: Patients with IgE-mediated CMA had higher specific IgE concentrations to casein compared with whey proteins ($P < 0.001$). In this group of patients, there was a positive correlation between IgE levels and levels of the other isotypes for all four milk proteins ($P < 0.001$). In general, the caseins were the more allergenic and antigenic proteins in all groups of patients. Patients with enterocolitis syndrome produced less milk protein-specific IgG₄ ($P < 0.05$) and had a trend for higher IgA antibody levels when compared to the control group. Lymphocyte proliferative responses in all groups with CMA were significantly higher than controls ($P < 0.05$), although this response was similar in patients with IgE- and non-IgE-mediated CMA.

Conclusion: There is a distinct pattern of humoral antibody response in the different forms of CMA. Patients with IgE-mediated CMA have an elevated polyisotypic response to cow milk protein. The relative lack of specific IgG₄ production in patients with enterocolitis syndrome may be involved in the pathogenesis of the disease. In general, caseins appear to be the predominant allergen in patients with CMA.

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Key words: allergy; antibodies; casein; children; α -lactalbumin; β -lactoglobulin.

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Accepted for publication 4 May 2004

Cow milk is one of the first foods introduced into an infant's diet, and cow milk allergy (CMA) is the most prevalent food allergy in the first year of life, affecting about 2.5% of infants (1, 2). Immunoglobulin (Ig)E-mediated allergy accounts for about 60% and non-IgE mediated hypersensitivity accounts for about 40% of adverse reactions to milk (3), with the majority of IgE-mediated reactions involving the skin, and the majority of non-IgE mediated reactions involving the gastrointestinal tract. In IgE-mediated food hypersensitivity, patients develop urticaria, atopic dermatitis (AD), vomiting,

diarrhea, shortness of breath, laryngeal edema and/or hypotension with cardiovascular collapse. The reason for the two forms of IgE-mediated skin reactions, urticaria and AD, are poorly understood. Urticaria represents a classic type I hypersensitivity reaction with allergen-induced cross-linkage of IgE on mast cells. AD also has been associated with IgE-mediated mast cell activation. In addition, an involvement of other cell types including T cells and Langerhans cells is well established. Among the various cow milk-induced, gastrointestinal hypersensitivities, cow milk-induced enterocolitis syndrome and

allergic eosinophilic gastroenteritis (AEG) have been described (4). Children with non-IgE-mediated food protein-induced enterocolitis syndrome present early in infancy with diarrhea, emesis and if prolonged, failure to thrive (5). Diagnosis is based upon the clinical history and confirmed if there is improvement when the causal protein is eliminated from the diet, followed by a positive challenge when the food is re-introduced (6). AEG affects all age groups and typically presents as postprandial nausea, vomiting, abdominal pain, diarrhea, early satiety or food refusal, and weight loss (7, 8). Diagnosis is confirmed by biopsy evidence of infiltration of the gastric or intestinal walls with eosinophils. Allergic eosinophilic gastroenteritis is considered a mixed IgE- and non-IgE-mediated disorder as some patients with this condition have detectable IgE antibodies to the food trigger (7).

The central role of IgE in food-induced IgE-mediated reactions has been well described. Less is known about the role of the other immunoglobulin classes. There is some evidence for increased milk-protein specific IgG₁ and IgG₄ (9), higher IgA, IgG₁ and IgG₄ antibodies to α -lactalbumin (10), and higher IgG₄ antibodies to β -lactoglobulin (11) in atopic children. These findings are not always consistent and some authors have found either no difference in IgA and IgG levels to cow milk protein (CMP) between CMA and controls (12, 13) or have even found lower IgG antibody levels in CMA patients (14). With regard to cellular responses, studies have shown that lymphocytes from patients with IgE-mediated CMA proliferate significantly more in the presence of milk antigens compared to control subjects (15, 16). However, other authors have shown no difference between individuals with and without CMA (17–19). These conflicting results have resulted in controversy as to the difference in proliferative response between allergic and nonallergic individuals. Much less is known about the immunopathogenesis of enterocolitis syndrome and AEG. There is evidence to suggest the involvement of T cells that produce T_H2 type cytokines when stimulated with milk proteins (20). Increased TNF- α from circulating milk protein-specific T cells (21) and decreased TGF- β 1 in intestinal mucosa (22) may also play a role in food-protein induced enterocolitis. However, virtually nothing is known about the humoral response to milk proteins in these disorders.

Milk proteins have been well characterized and divided into casein and whey fractions (ratio 80 : 20). α -Casein, β -casein, κ -casein, and the whey proteins α -lactalbumin and β -lactoglobulin account for most of the protein found in milk (42, 25, 10, 9 and 4% respectively) (23). There is still disagreement about the relative antigenicity of these different proteins. The contribution of the different immunoglobulin classes to different CMPs in the various disorders of CMA has also not been investigated.

In this study, we evaluated humoral (IgE, IgA, IgG₁ and IgG₄) and cellular responses to α -casein, β -casein, κ -casein, α -lactalbumin and β -lactoglobulin in patients with milk-induced IgE-mediated disorders, enterocolitis syndrome and AEG, as well as in control subjects.

Materials and methods

Patients

Patients were recruited from among those referred to the pediatric allergy clinic at The Mount Sinai Medical Center, New York. The first group consisted of 113 patients with IgE-mediated CMA (age range 6–264 months, median 67 months; 69% male). The diagnosis was based either on a convincing history of an allergic reaction after isolated ingestion of milk ($n = 72$), or in ambiguous cases, a double-blind, placebo-controlled food challenge (DBPCFC) was performed ($n = 41$). Fifty-two patients had only cutaneous symptoms consisting of either urticaria or AD and 53 had cutaneous symptoms with either respiratory or gastrointestinal complaints. The remaining cases involved other organ systems without cutaneous involvement. The second group included 11 patients with milk-protein induced enterocolitis syndrome (age range 7–250 months, median 41 months; 36.4% males). Milk-protein induced enterocolitis syndrome was diagnosed if the patient fulfilled the criteria of Powell (5) in the absence of detectable milk-specific serum IgE. All patients were symptomatic before the age of 7 months, although for some, their sera were obtained at a later time point. Six (55%) had the diagnosis confirmed by DBPCFC. The third group included 16 patients with AEG (age range 12–211 months, median 63 months; 62.5% males). All patients had the typical symptoms and biopsy evidence of eosinophilic infiltration of the gastrointestinal tract, as previously described (8). In all three groups, serum used for analysis was obtained at the time of clinical reactivity. Normal controls were recruited from patients referred for evaluation of allergic disease who had clinical tolerance to milk and no evidence of food allergy. This group consisted of 21 individuals (age range 8–221 months, median 56 months; 61.9% males). Except for significantly fewer males in the enterocolitis group when compared with the IgE-mediated group, there were no significant differences when comparing the ages or sexes of the various groups.

Informed consent was obtained, and the study approved by the Mount Sinai School of Medicine Institutional Review Board.

Determination of milk-specific immunoglobulin levels

Cow milk specific IgE levels (in kU/l) were measured with the CAP-System FEIATM (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's recommendation.

Serum IgE, IgA, IgG₁ and IgG₄ antibody levels to α -casein, β -casein, κ -casein, α -lactalbumin and β -lactoglobulin were determined using enzyme-linked immunosorbent assay. Genetically engineered human-mouse chimeric antibodies specific for 4-hydroxy-3-nitrophenyl acetic acid and nitrophenyl-o-caproic acid succinimide (NP) coupled to human serum albumin (HSA) (courtesy of R Hamilton, Ph.D., Johns Hopkins University, Baltimore, MD) were used to generate standard curves. The upper two rows of 96-well microtiter plates were coated with NP-HSA and the lower wells with the individual cow milk proteins (Sigma chemical, St Louis, MO) at 100 ng/well in carbonate buffer, pH 9.0 and incubated overnight at 4°C. Plates were washed and blocked with 1% HSA in PBS with 0.05% Tween 20

(PBS-T), and then known quantities of chimeric IgE, IgA, IgG₁ or IgG₄ anti-NP (all courtesy of R Hamilton, Johns Hopkins University) were added to the upper two rows to generate a standard curve. A separate plate was used for each antibody subclass. Simultaneously, dilutions of sera from patients were added in duplicate to the lower wells. Sera were diluted 1 : 10 for IgE and IgA measurements, and either 1 : 10 or 1 : 100 for IgG₁ and IgG₄ measurements. Plates were incubated overnight at 4°C. After washing, the plates were developed using standard protocol. Milk-protein specific antibody determinants were compared with the standard curve, and antibody values reported as NP-equiv-
alent units. The lower limit of detection of the assays were as follows: IgE – 10 ng/ml, IgA – 40 ng/ml, IgG₁ – 0.1 µg/ml and IgG₄ – 40 ng/ml.

Determination of milk-specific lymphocyte proliferative response

Heparinized venous blood drawn in a sterile manner was obtained from a subset of these patients (83 with IgE-mediated CMA, 11 with enterocolitis, seven with AEG and 10 controls). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation and proliferation assays performed as previously published (24). Briefly, PBMCs were cultured in triplicate with or without milk protein in 96-well flat-bottom plates. We have previously established optimal concentrations for milk-allergen specific proliferation (24). Therefore, caseins were used at 200–500 µg/ml and whey proteins at 5–50 µg/ml. Each well contained 1 × 10⁵ PBMCs in serum-free culture medium (AIM-V with L-glutamine, penicillin and streptomycin, all GIBCO laboratories, Grand Island, NY). After 5 days, cells were pulsed with 1 µCi of tritiated thymidine (ICN Biomedicals, Irvine, CA) per well, harvested 16 hours later, and counted in a liquid scintillation counter. Results were expressed as the stimulation index (SI), which is the mean cpm for antigen-stimulated cells divided by the mean cpm for unstimulated cells.

Statistics

Data between groups were analyzed with the nonparametric Mann–Whitney *U*-test and correlations with Spearman’s rank order correlation coefficient test. Calculations were performed with SPSS 11.0 for windows (SPSS Inc, Chicago, IL) and graphs drawn with GraphPad Prism 4.0 for windows (GraphPad Software, San Diego, CA). *P*-values <0.05 were considered significant.

Results

Total milk specific IgE

As expected, patients with milk-induced IgE-mediated hypersensitivity had significantly higher milk-specific IgE levels (median 16.1 kU/l) when compared with the other groups (Fig. 1). Three patients in this group had no detectable milk-specific IgE but had challenge-proven CMA with positive prick skin tests to cow milk. Ten of 16 patients with AEG had detectable specific IgE antibodies to cow milk (Table 1). The median milk-specific IgE for the whole AEG group was 1.1 kU/l (Fig. 1). None of the patients with enterocolitis syndrome or the controls had detectable milk-specific IgE.

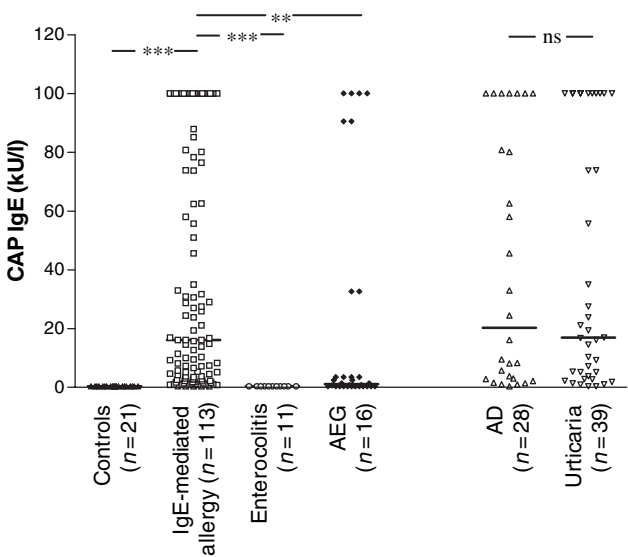


Figure 1. Milk-specific immunoglobulin (Ig)E levels (kU/l) measured with the CAP-System FEIA in controls, patients with cow milk-induced IgE-mediated allergy, food protein-induced enterocolitis, allergic eosinophilic gastroenteritis, atopic dermatitis (AD) and urticaria. Note that the latter two groups are a subset of the patients with IgE-mediated allergy. The lower and upper limits of detection of this assay are 0.35 and 100 kU/l respectively. ****P* < 0.001, ***P* < 0.01, ns, not significant.

Antigenicity and allergenicity of casein and whey proteins

The caseins were the more allergenic (IgE binding) and antigenic (IgA and IgG binding) proteins for all groups of patients with CMA (Table 1, Fig. 2A–D). Among patients with IgE-mediated reactions, 64.6% had detectable IgE to α-casein, 75.2% to β-casein, 47.8% to κ-casein and 36.2% for both α-lactalbumin and β-lactoglobulin (Table 1). In addition, for this group, the levels of IgE-antibodies to α- and β-casein were significantly higher than to the whey proteins (*P* < 0.001, Fig. 2A). In these patients, levels of IgA, IgG₁ and IgG₄ antibodies to α- and β-casein were also higher compared with the whey proteins (*P* < 0.001, Fig. 2B–D). Median anti-κ-casein antibody levels were also higher than that to the whey

Table 1. Number of patients with detectable immunoglobulin (Ig)E by either CAP-System FEIA™ or enzyme-linked immunosorbent assay (% of total)

	Control (n = 21)	IgE-mediated allergy (n = 113)	Enterocolitis (n = 11)	AEG (n = 16)
CAP-FEIA	0	110 (97.3)	0	10 (62.5)
α-Casein	0	73 (64.6)	1 (9.1)	4 (25.0)
β-Casein	0	85 (75.2)	4 (36.4)	5 (31.2)
κ-Casein	0	54 (47.8)	0	4 (25.0)
α-Lactalbumin	0	41 (36.2)	0	3 (18.7)
β-Lactoglobulin	0	41 (36.2)	1 (9.1)	3 (18.7)

AEG, allergic eosinophilic gastroenteritis.

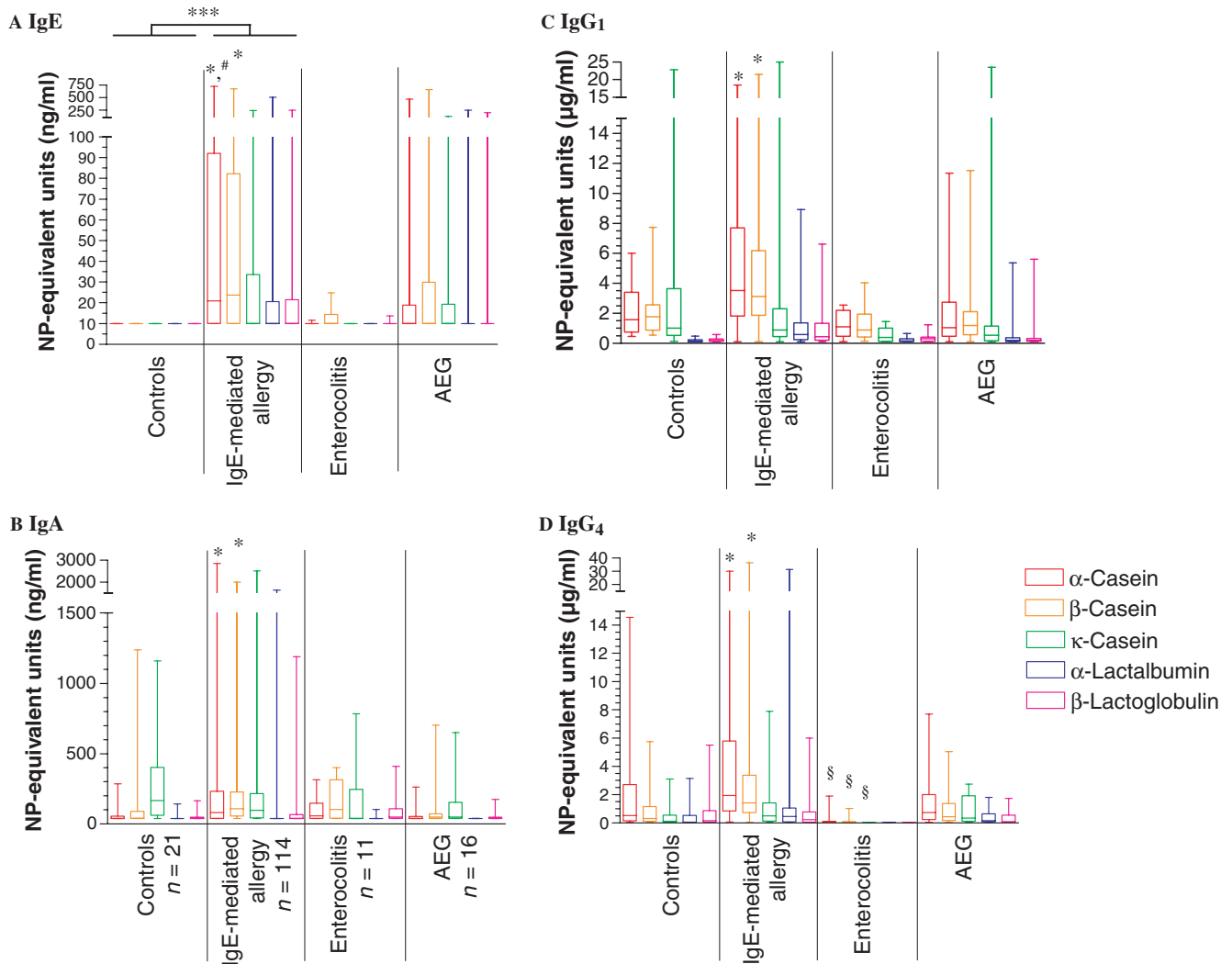


Figure 2. Milk protein-specific immunoglobulin (Ig)E (A), IgA (B), IgG₁ (C) and IgG₄ (D) antibody levels measured by enzyme-linked immunosorbent assay. Boxes represent the 25th, 50th and 75th percentiles and whiskers the lowest and highest values. *** $P < 0.001$ when each of the five milk proteins is compared between the control and the IgE-mediated group. * $P < 0.001$ when α - and β -casein is each compared individually with α -lactalbumin and β -lactoglobulin within the group. # $P < 0.05$ when compared with α -casein-specific IgE in the enterocolitis group. § $P < 0.05$ when compared with the corresponding milk protein-specific IgG₄ in the control group.

proteins, but the difference was not statistically significant. The caseins showed a general trend to be more antigenic for all isotypes in all study groups. α -Lactalbumin was the least allergenic and antigenic of the five proteins tested, with 36.2%, 0% and 18.7% of patients with IgE-mediated reactions, enterocolitis and AEG respectively having detectable IgE to this protein (Table 1). The IgE antibodies to the individual CMPs were undetectable in the control group. There were four patients with enterocolitis who had detectable IgE antibodies to β -casein and one patient with detectable IgE antibodies to both α -casein and β -lactoglobulin, despite the absence of milk-specific IgE detectable by CAP-System FEIA.

Humoral response to milk proteins in IgE- and non-IgE-mediated CMA

Patients with IgE-mediated CMA had significantly higher levels of specific IgE for all milk proteins compared with the controls and higher α -casein antibody concentrations compared with the enterocolitis group (Fig. 2). Also, for the IgE-mediated group, there was a significant positive correlation between IgE levels and levels of the other immunoglobulin isotypes for all milk proteins ($P < 0.001$, Fig. 3A–C, only data for α -casein shown). The tendency of individuals with higher milk-specific IgE to have higher milk-specific IgA, IgG₁ and IgG₄, i.e. a polyisotypic immunoglobulin response, was also seen in

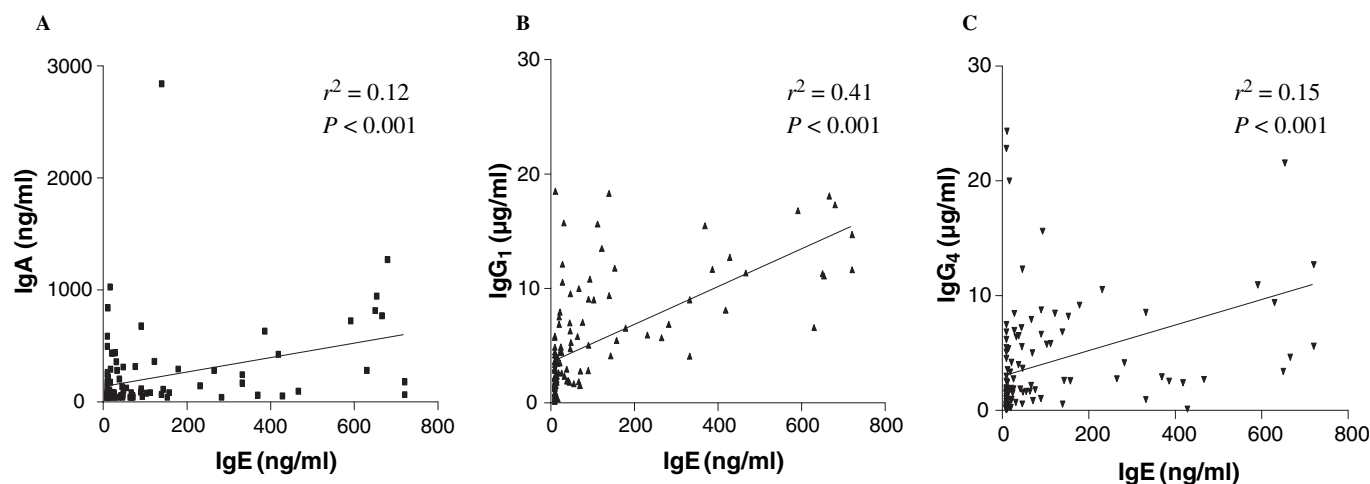


Figure 3. α -Casein specific-immunoglobulin (Ig)A (A), IgG₁ (B) and IgG₄ (C) levels plotted against α -casein specific-IgE levels for patients with IgE-mediated allergy.

patients with AEG. Patients with enterocolitis syndrome produced significantly less IgG₄ antibodies to caseins when compared with the controls (Fig. 2D), with a similar trend seen for whey proteins without having increased IgG₁ levels (Fig. 2C).

We performed a subgroup analysis of patients who had either AD or urticaria to see if there was any difference between antigen handling and immunoglobulin production. There was no difference between these groups in total milk-specific IgE (Fig. 1) or in immunoglobulin isotype production to any of the five individual CMPs (Fig. 4).

Lymphocyte proliferative responses to milk proteins

Overall, proliferative responses to all three caseins were significantly higher than to whey proteins ($P < 0.001$). For patients with CMA, median SI for α -casein, β -casein and κ -casein was 5.0 (range 0.4–215.9), 6.3 (range 0.5–270.5) and 7.2 (range 0.3–280.2) respectively while the median SI for α -lactalbumin was 1.9 (range 0–143.6) and for β -lactoglobulin 1.7 (0–71.3). Patients from all groups with CMA had significantly higher proliferative responses to milk proteins than normal controls (Fig. 5). There was no significant difference between the three groups with CMA or between patients with AD and urticaria.

Discussion

In the present study we characterized the humoral and cellular response to five major cow milk proteins in patients with IgE- and non-IgE-mediated CMA. We found that caseins were the predominant allergenic

and antigenic CMP in patients with cow milk-induced IgE-mediated reactions, consistent with findings from previous studies (25, 26). A criticism of our results may be that not all the patients with IgE-mediated CMA had challenge-proven milk allergy. In a further analysis, we therefore included in this group only patients with challenge-proven allergy or with milk-specific IgE above 15 kU/l (the level above which a patient has a 95% likelihood of clinical reactivity) (27). The results of this analysis were comparable with results obtained with all IgE-mediated subjects, described above.

The production of IgA and IgG antibodies to CMP is a normal physiological response to the ingestion of milk (28). However, our patients with cow milk-induced IgE-mediated reactions produced significantly more milk-specific antibodies than the controls although they all maintained a milk-free diet. This is in agreement with previous studies (25, 29, 30). We further demonstrated that the polyisotypic response to CMP in patients with CMA is proportional. This positive correlation between specific IgE and IgA/IgG₁/IgG₄ to CMP has been previously shown in patients with AD (9). The same trend of a proportional polyisotypic response has been reported in patients with allergy to peanut (31) and aeroallergens (10, 32). These isotypes are unlikely to be pathogenic as they were found in the control group of patients who tolerate cow milk. The increased levels of milk-specific antibodies in patients with CMA may be a result of increased gut permeability resulting in greater passage of antigen into the circulation and the generation of a more robust antibody response (33). It also may be a reflection of the Th2 bias in allergic individuals, as Th2 cytokines such as IL-4 are more efficient inducers of immunoglobulin synthesis (34). We also compared the humoral and cellular response to CMP between patients

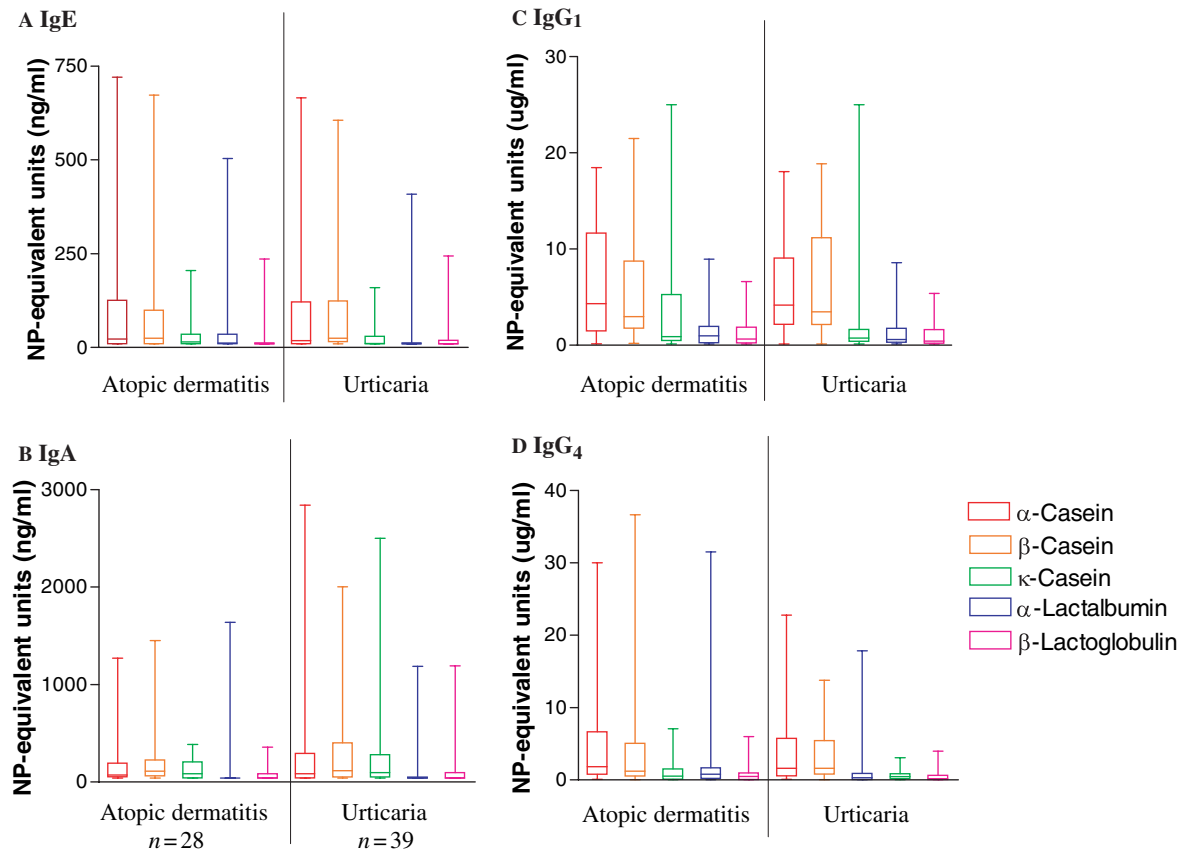


Figure 4. Milk protein-specific immunoglobulin (Ig)E (A), IgA (B), IgG₁ (C) and IgG₄ (D) antibody levels measured by enzyme-linked immunosorbent assay in patients with atopic dermatitis or urticaria. No significant difference between the two groups.

with AD and acute urticaria. The reasons for these two distinct IgE-mediated reactions involving the skin are poorly understood. It is well established that T-cells expressing Th2 cytokines play an important role in AD (35). The humoral response in patients with AD might be influenced by these cytokine-releasing cells and differ from patients with milk-induced urticaria. We have shown that lymphocytes expressing the skin homing receptor CLA may also play an important role in the pathogenesis of milk-induced urticaria (36). However, the present study showed no difference in either humoral or lymphoproliferative responses between these two distinct milk-induced skin reactions.

Little is known about the humoral response in patients with milk-protein induced gastrointestinal syndromes. Similar to the IgE-mediated CMA group, we found that caseins were the predominant antigenic and allergenic CMP in patients with enterocolitis and AEG. Elevated serum IgA and IgG antibodies to food proteins have been described in patients with food protein-induced enterocolitis (37). We found a similar elevation of milk protein-specific IgA in our patients with this syndrome. However, we did not find the IgG₁ or IgG₄ antibodies to

be elevated compared with controls. In fact, patients with enterocolitis syndrome produced almost no milk protein-specific IgG₄ antibodies. IgG₄ antibodies fix complement poorly, and could have a protective role in competing with other subclasses that could activate complement. This relative lack of IgG₄ in patients with enterocolitis may be involved in the pathogenesis of the disease. In addition, a subset of patients with enterocolitis syndrome had detectable IgE antibodies to three of the individual milk proteins, which was not seen when measuring IgE levels against whole cow milk proteins. Some patients with this syndrome have been found to have detectable but low levels of IgE antibodies to the responsible food. It has been suggested that this could be a marker of persistent hypersensitivity (6). Further work is required to determine if these patients recognize the same epitopes on CMP as patients with IgE-mediated hypersensitivity.

We have shown that the lymphocyte proliferative response in patients with CMA is significantly higher than in controls, demonstrating the presence of a milk protein-specific lymphocyte response. Previous studies have shown that the functional phenotype of these cells

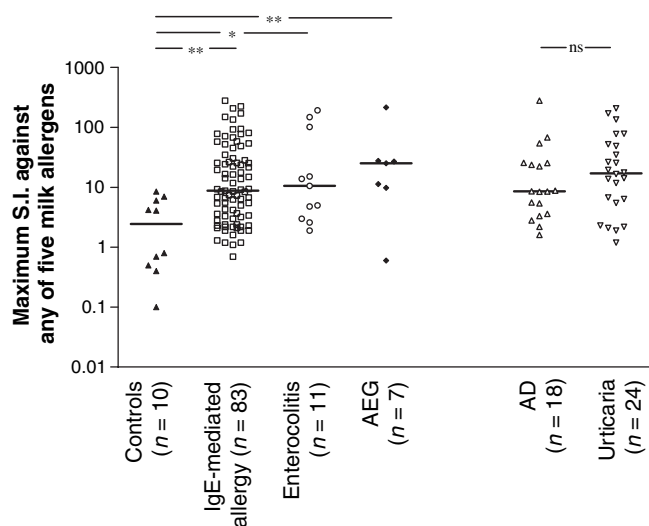


Figure 5. Lymphocyte proliferation to milk proteins in controls, patients with immunoglobulin (Ig)E-mediated allergy, food protein-induced enterocolitis, AEG, AD and urticaria. Shown is the maximum proliferative response to any of the five milk allergens for individual patients. Lines represent the median value. ** $P < 0.01$, * $P < 0.05$, ns, not significant.

have a T_H2 skew (20, 38). However, the proliferative response from patients with IgE- and non-IgE-mediated CMA was not significantly different. Thus, proliferation assays with PBMCs cannot be used to distinguish between the different forms of CMA. The difference may lie in the microenvironment, especially with local cytokines secreted (39). The relevance of CMP-specific T cells in peripheral blood, their homing to organs of disease, and the resultant cytokine secretion therein needs to be investigated.

In summary, we were able to show that patients with IgE- and non-IgE-mediated CMA show a distinct humoral response to individual cow milk proteins with a polyisotypic elevation in patients with IgE-mediated reactions and a lack of milk-specific IgG₄ antibodies in patients with enterocolitis syndrome. In all milk-induced hypersensitivity disorders, caseins appeared to be the most predominant allergenic and antigenic cow milk proteins. There is a cow milk protein-specific cellular response in patients with CMA, but this was similar between the different forms of CMA.

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