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ORIGINAL CONTRIBUTION

Specific antibodies to cow's milk proteins in infants: effect of early feeding and diagnosis of cow's milk allergy

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Abstract
Purpose To investigate whether specific IgA, IgG, IgG1 and IgG4 responses to cow's milk proteins differ between infants with cow's milk allergy and infants with cow's milk related symptoms (control subjects), and whether early feeding affects these responses as well as specific IgE.
Methods A cohort of 6,209 healthy, full-term infants in a double-blind randomized trial received, as supplementary feeding at maternity hospitals (mean duration 4 days), either cow's milk formula, extensively hydrolyzed whey formula or donor breast milk. Infants who developed cow's milk associated symptoms ($n = 223$) underwent an open oral cow's milk challenge (mean age 7 months), which confirmed cow's milk allergy in 111 and was negative in 112. We measured in sera cow's milk specific IgE levels with UniCAP (Phadia, Uppsala, Sweden), and β-lactoglobulin and α-casein specific IgA, IgG1, IgG4 and IgG levels with enzyme-linked immunosorbent assay.
Results Infants with IgE-mediated cow's milk allergy had lower β-lactoglobulin and α-casein specific IgG1, IgG4 and IgG levels ($p < 0.05$) than infants with non-IgE-mediated cow's milk allergy or control subjects. Within the group of infants with cow's milk allergy, exposure to cow's milk during the first few days after birth led to higher β-lactoglobulin and α-casein specific IgG4 levels ($p < 0.005$) compared to infants fed with either breast milk or extensively hydrolyzed formula.
Conclusions Subdued IgG class responses to cow's milk proteins characterized IgE-mediated cow's milk allergy. In infants who developed cow's milk allergy early exposure to cow's milk resulted in a heightened specific IgG4 response.

Keywords α-Casein · β-Lactoglobulin · Cow's milk allergy · Infants' feeding · IgG4 · Tolerance

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Introduction

The first oral antigen that infants in Western countries encounter is commonly cow's milk (CM). It elicits an immune response in all infants, whereas only few (2–3%) develop cow's milk allergy (CMA) [1]. The timing of first exposure influences the immune response [2–5].

Humoral response to food antigens predominantly consists of class IgG and IgA antibodies [6], while high production of specific IgE antibodies often leads to allergy. Recent research suggests that development and maintenance of clinical tolerance involves IgG4 antibodies [7, 8]. High levels of IgG4 to β-lactoglobulin and/or ovalbumin as well as a high specific IgG4/IgE ratio predicted early clinical tolerance in CM and/or egg allergy [9]. In addition to IgG4, specific IgA levels in serum reportedly increased during successful grass pollen immunotherapy [8].

Early exposure to CM may or may not associate with risk for CMA [1, 4]. Increased IgG class responses have, however, been reported following exposure to CM proteins during first 3 days [3] or 3 months [2, 4] of life.

We investigated whether specific antibody responses to CM proteins at a mean age of 7 months differed between infants diagnosed with CMA ($n = 111$) and those with CM related symptoms but negative oral CM challenge (control subjects, $n = 112$). We furthermore studied in a double-blind, randomized trial how CM exposure during the first days of life affects these antibody responses.

Methods

Study population

We examined serum samples from 223 infants at mean age 7 months (±SD 2). Informed and written consent was received from infants' parents. The Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study.

A population-based cohort of 6,209 full-term newborn infants in a prospective, double-blind, randomized trial received either liquid CM formula (Tutteli, Valio, Finland), extensively hydrolyzed whey formula (Pepti-Junior, Nutricia, The Netherlands) or donor BM (a mixture of BM from multiple donors expressed 1–6 months after delivery) at maternity hospital after birth (mean 4 days) [1]. The current study involved infants ($n = 223$) with cow's milk related symptoms which appeared at the mean age of 2.9 months and subsided rapidly within days on an elimination diet (mean time, 5 days). They underwent, at mean age 7 months, (±SD 2) an open oral CM challenge, which confirmed CMA in 111 [1]. Of 111 infants with CMA, 82 had IgE-mediated and 29 non-IgE-mediated CMA. We defined IgE-mediated CMA based on positive CM skin prick test (wheal diameter ≥3 mm greater than the negative control) or CM specific IgE antibodies (≥0.7 kU/L measured with UniCap, Phadia, Uppsala, Sweden) or both any time 0–12 months after CMA diagnosis [10]. We designated infants who reacted in the oral CM challenge but had no detectable CM specific IgE reaction as having non-IgE-mediated CMA. In those 29 infants, oral CM challenge elicited eczema (70%), urticaria (6.7%), pruritus (23%), vomiting (33%), diarrhea (20%) and rhinitis (10%). We designated infants with cow's milk related symptoms but negative oral CM challenge as control subjects ($n = 112$). Previous studies provided clinical data [1, 5, 10]. Duration of breast feeding and the introduction of foods were comparable in the groups divided by CMA diagnosis or supplementary feeding [5].

Measurement of antibodies

We stored serum samples at −80 °C until analyzed. We measured CM specific IgE antibodies in sera with enzymatic UniCap fluoroimmunoassay (Phadia, Uppsala, Sweden) [5].

We applied enzyme-linked immunosorbent assays (ELISA) as previously described [11] for measuring bovine-β-lactoglobulin, bovine-α-casein and ovalbumin specific IgA and IgG levels, and for IgG1 and IgG4 of same specificity we adapted ELISA protocols from Ruiter et al. [7]. We used horseradish peroxidase (HRP) conjugated anti-human IgG4 (Sanquin, Amsterdam, Netherlands) or anti-human IgG1 (Zymed Labotatories, San Fransisco, CA, USA). Antibody levels were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with high level of antibodies after subtracting the blanks.

Statistical analysis

We compared logarithmic transformations of antibody levels with one-way analysis of variance between the three diagnosis groups (IgE-mediated, non-IgE-mediated and control subjects), as well as between feeding groups within subjects with or without CMA diagnosis. Infants randomized to receive donor BM and infants fed exclusively with mother's BM were combined in the BM group. For pairwise comparisons, we applied independent samples t test. We considered a p value of 0.05 or less statistically significant. We performed the analysis with SPSS 15.0.1.

Results

Infants with IgE-mediated CMA had lower IgG1 and IgG4 levels to CM proteins than infants with non-IgE-mediated CMA or control subjects

Infants with IgE-mediated CMA had lower specific IgG1 and IgG4 levels to β-lactoglobulin and to α-casein, as well as IgG to α-casein, than infants with non-IgE-mediated CMA or control subjects (Table 1). Specific IgA levels to β-lactoglobulin and α-casein showed no difference between groups, except that in pairwise comparison IgE-mediated CMA group had lower levels of α-casein IgA than control group (Table 1). In contrast to CM specific antibodies, specific IgG, IgG1 and IgA levels to ovalbumin were highest in the IgE-mediated CMA group (Table 1). CM specific IgE levels were highest in infants with IgE-mediated CMA while the levels in infants with non-IgE-mediated CMA did not differ from control subjects (Table 1). Levels of IgA to β-lactoglobulin and α-casein, IgG to β-lactoglobulin and IgG4 to ovalbumin showed no significant difference between the groups (data not shown).

Exposure to CM in maternity hospital was associated with higher CM specific antibody levels in infants later diagnosed with CMA

Feeding during the first few days of life in maternity hospitals was associated with CM specific humoral responses at a mean age of 7 months in infants diagnosed with CMA. Among them, those fed with CM formula had higher

Table 1 Specific antibody levels to cow's milk (CM), β-lactoglobulin (BLG), α-casein (CAS) and ovalbumin (OVA) in infants with IgE-mediated or non-IgE-mediated cow's milk allergy (CMA), and control subjects

	IgE-mediated CMA, $n = 82$ [Geometric mean (95% confidence interval)]	Non-IgE-mediated CMA, $n = 29$ [Geometric mean (95% confidence interval)]	Control, $n = 112$ [Geometric mean (95% confidence interval)]	p one-way analysis of variance
BLG IgG1 (AU)	1.0 (0.7–1.6)	2.2 (1.2–4.2)	**1.7 (1.3–2.3)**	0.05
CAS IgG1 (AU)	3.5 (2.4–5.0)	**9.1 (5.0–16.5)**	**7.2 (5.7–9.2)**	0.001
BLG IgG4 (AU)	4.4 (2.8–7.0)	**13.2 (5.7–30.8)**	**9.9 (6.5–15.2)**	0.02
CAS IgG4 (AU)	2.5 (1.6–3.9)	2.1 (1.0–4.4)	**6.5 (4.6–9.4)**	0.001
CAS IgG (AU)	1.9 (1.4–2.7)	**4.2 (2.4–7.4)**	**4.3 (3.1–5.8)**	0.002
OVA IgG1 (AU)	25.2 (14.9–42.6)	8.8 (3.3–23.7)	**11.1 (7.6–16.2)**	0.02
OVA IgG (AU)	14.2 (8.8–23.1)	**2.8 (1.0–8.0)**	**4.7 (2.9–7.6)**	0.002
OVA IgA (AU)	13.6 (8.0–23.3)	**3.2 (1.7–6.3)**	7.7 (5.1–11.7)	0.01
CM IgE (kU/L)	1.8 (1.3–2.6)	**0.2 (0.2–0.3)**	**0.2 (0.1–0.3)**	<0.0001

AU designates arbitrary units

Text in bold denotes that t test p value < 0.05 in pairwise comparison with the group of IgE-mediated cow's milk allergy

specific IgE levels (geometric mean 1.4 kU/L; 95% confidence interval 0.8–2.4; $n = 40$; t test $p < 0.03$) than infants fed with HF (0.58 kU/L; 0.4–0.9; $n = 25$). They furthermore had higher levels of β-lactoglobulin and α-casein specific IgG4 levels than infants fed with either BM or HF (Table 2). For IgG4 levels to β-lactoglobulin, the difference also reached statistical significance in the subgroup of infants with IgE-mediated allergy (Table 2). In the CMA group, α-casein specific IgA levels were highest among infants fed with CM formula, particularly compared with HF group (Table 2).

Discussion

We report that at a mean age of 7 months, infants with IgE-mediated CMA had lower specific IgG1 and IgG4 levels to CM proteins than infants with non-IgE-mediated

Table 2 Specific IgG4 levels to β-lactoglobulin (BLG) and α-casein (CAS), and specific α-casein IgA levels as arbitrary units (AU) in infants with cow's milk allergy (CMA), further divided to IgE-mediated CMA and non-IgE-mediated CMA, and control subjects, grouped according to the supplementary feeding at maternity hospital

	Cow's milk formula	Hydrolyzed formula	Breast milk[a]	p one-way analysis of variance
BLG IgG4 [geometric mean (95% confidence interval) AU; n]				
CMA	15.2 (7.2–32.0); 38	5.8 (2.6–13.3); 25	**2.8 (1.6–4.8); 48**	0.002
IgE-mediated CMA	11.6 (5.3–25.3); 32	4.4 (1.6–12.2); 17	**1.8 (1.0–3.0); 33**	0.001
Non-IgE-mediated CMA	62.6 (7.9–498.2); 6	10.5 (2.5–44.4); 8	8.0 (2.6–24.5); 15	0.2
Controls	14.9 (7.0–32.0); 40	13.5 (6.0–30.5); 27	9.9 (3.1–10.8); 45	0.1
CAS IgG4 [geometric mean (95% confidence interval) AU; n]				
CMA	5.4 (2.5–11.4); 38	**1.1 (0.56–2.17); 25**	**1.8 (1.1–3.0); 48**	0.005
IgE-mediated CMA	4.5 (2.1–10.0); 32	1.4 (0.5–3.5); 17	1.9 (1.0–3.5); 33	0.1
Non-IgE-mediated CMA	13.7 (1.5–127.7); 6	**0.7 (0.4–1.4); 8**	1.7 (0.7–4.2); 15	0.02
Controls	11.5 (6.2–21.4); 41	5.7 (3.0–11.1); 28	**4.3 (2.5–7.6); 47**	0.06
CAS IgA [geometric mean (95% confidence interval) AU; n]				
CMA	1.7 (1.0–2.7); 38	**0.7 (0.4–1.1); 25**	1.4 (0.9–2.1); 48	0.04
IgE-mediated CMA	1.6 (1.0–2.6); 32	**0.7 (0.3–1.5); 17**	1.0 (0.6–1.6); 33	0.1
Non-IgE-mediated CMA	2.0 (0.2–16.2); 6	0.6 (0.3–1.3); 8	3.1 (1.7–5.5); 15	0.06
Controls	2.1 (1.4–3.3); 41	2.1 (1.3–3.3); 28	1.4 (1.0–2.0); 47	0.2

[a] Includes both infants randomized to receive donor breast milk and infants who received exclusively mother's breast milk

Text in bold denotes that t test p value < 0.05 in pairwise comparison with the cow's milk formula group within the diagnostic group

CMA or with negative oral CM challenge. We furthermore show that exposure to CM during the first few days of life was associated with heightened levels of CM specific IgE as well as of β-lactoglobulin and α-casein specific IgG4 7 months later in the subgroup of all infants who were diagnosed with CMA as well as in the further subgroup of those with IgE-mediated CMA. The population was part of a double-blind, randomized trial in healthy, full-term infants who received either BM only, HF or CM formula during first 4 days of life and were followed for the emergence of CMA [1].

The observed combination of high IgE and low IgG4 levels to CM proteins in infants with CMA confirms that the balance between allergen specific IgE and IgG4 has an impact on whether clinical allergy or tolerance develops [7–9]. The finding that infants with IgE-mediated CMA had pronounced IgG class responses to ovalbumin suggests that their subdued IgG1 and IgG4 responses to CM proteins were antigen specific rather than resulting from a compromised IgG class response to oral antigens in general. It furthermore insinuates that the differences between study groups in humoral responses to oral antigens did not ensue from disparities in gut permeability.

Our data confirm previous reports that early exposure to CM, even briefly, results in heightened humoral responses to CM proteins [2–4]. We observed an association of CM protein specific IgG4 and IgA levels with early CM exposure only in infants with CMA. A minority of these infants showed symptoms, predominantly eczema, in the oral CM challenge without signs of specific IgE reaction to CM. The majority, however, developed an IgE-mediated reaction to CM. They plausibly had a propensity for more intense and Th2-skewed immunological responses to oral antigens. While Th2-cells induce both IgE and IgG4 production [12], upregulation of IgG4 production by IL-10 is related to the development and maintenance of tolerance [8]. Also IgA may counterbalance IgE-mediated responses [8]. Although early exposure to CM in infants susceptible to atopy promoted the production of not only CM specific IgE, but also of specific, plausibly tolerogenic IgG4 and IgA, the balance shifted to allergic reactivity rather than tolerance.

We demonstrated that infants with IgE-mediated CMA while having heightened CM specific IgE levels concomitantly showed subdued specific IgG4 levels, reflecting a failure in tolerance induction. Furthermore, early exposure to CM resulted in increased humoral responses to CM proteins among infants who developed CMA. Our study thus underscores the balance between IgE and IgG class antibodies in oral tolerance, and how it intertwines with environmental exposure and atopic predisposition.

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