

Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins

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Background: Cow's milk allergy (CMA) affects 2.5% of young infants. In previous murine studies it was observed that allergic sensitization to the major cow's milk allergens casein and whey led, respectively, to IgE-independent and IgE-dependent clinical responses.

Objectives: In this study the involvement of immunoglobulin free light chains (Ig-fLCs) in the hypersensitivity response to cow's milk proteins was explored in mice, and Ig-fLC serum levels were determined in children affected by CMA or atopic dermatitis (AD).

Methods: Mice were orally sham, casein, or whey sensitized. Acute allergen-specific skin responses were determined, and serum immunoglobulin and Ig-fLC concentrations were measured. Ig-fLC dependency was validated by using the Ig-fLC blocker F991 in actively and passively sensitized mice. Ig-fLC serum concentrations were measured in a cohort of infants with CMA and infants with AD.

Results: After sensitization, no specific IgE was detectable in sera of casein-sensitized mice, whereas specific IgE levels were enhanced in whey-sensitized mice. Instead, Ig-fLC levels were increased in sera from casein-sensitized mice. Furthermore, blocking Ig-fLCs strongly diminished the allergic skin responses not only in casein-sensitized mice but also in mice transferred with splenocyte supernatants of casein-sensitized mice. In both patients with CMA and patients with AD, serum Ig-fLC concentrations were significantly enhanced.

Conclusions: This study indicates that sensitization with cow's milk proteins can lead to both IgE-dependent and Ig-fLC-dependent allergic hypersensitivity responses. Also, in children

affected with CMA or AD, serum Ig-fLC concentrations were increased, implying the relevance of Ig-fLC measurements in the diagnoses of human allergic disease. (*J Allergy Clin Immunol* 2010;■■■■:■■■-■■■.)

Key words: Immunoglobulin free light chains, IgE, cow's milk allergy, food allergy, casein, whey, atopic dermatitis

Cow's milk allergy (CMA) is a complex disorder, arising early in life and affecting 2% to 5% of children in the Western world. CMA is also associated with an increased risk of asthma later in life.^{1,2} The majority of patients with CMA are allergic to the major cow's milk proteins (CMPs) casein, whey, or both.^{3,4} Usually, reactions to a skin prick test are measured, titers of cow's milk-specific IgE are analyzed, or both in a patient's sera. However, there are patients with CMA who exhibit acute clinical features of CMA without detectable titers of specific IgE.^{3,5,6} Therefore the gold standard for diagnosis is still an orally induced double-blind, placebo-controlled food challenge (DBPCFC).⁷⁻¹⁰ The main disadvantages of a DBPCFC are the time-consuming character of the test, its costs, and the potential of inducing a severe allergic reaction. Although the incidence of anaphylaxis is low, its potential risk is quite stressful for the patient and his or her parents.⁷ Therefore the search for another clinical tool to diagnose CMA is ongoing. Thus far, no (immuno)therapy is available, and avoidance of cow's milk and the use of hydrolyzed formulae are currently the only effective strategies to prevent CMA symptoms.¹⁰

Previously, two preclinical models for CMA have been introduced in which mice were sensitized orally for whey or casein.¹¹ In these models the acute allergic skin reaction was monitored as a possible equivalent of the skin prick test. In both models all mice exhibit enhanced ear swelling on intradermal allergen challenge, which reflects systemic sensitization. The whey model resembles a typical type I allergy with high levels of whey-specific IgE and IgG₁. However, despite experiencing a pronounced acute allergic skin reaction on local allergen challenge, the response to casein was not associated with detectable levels of casein-specific IgE. Although the casein-sensitized mice did have enhanced specific titers of IgG₁, this was found not to correlate quantitatively with the skin reaction.¹¹ Therefore an alternative explanation for the induction of the acute allergic skin reaction was explored. In previous studies it was demonstrated that antigen-induced acute allergic responses could be elicited through immunoglobulin free light chains (Ig-fLCs).¹²⁻¹⁴ For instance, transfer of

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Abbreviations used

AD:	Atopic dermatitis
AU:	Arbitrary units
CMA:	Cow's milk allergy
CMP:	Cow's milk protein
DBPCFC:	Double-blind, placebo-controlled food challenge
fLC:	Free light chain
IQR:	Interquartile range

trinitrophenol-specific Ig-fLCs into naive mice sensitized them to the respective antigen. Local challenge of the passively sensitized animals with the appropriate antigen resulted in the induction of mast cell degranulation, leading to local inflammation. This Ig-fLC-elicited hypersensitivity response can be inhibited by local or systemic application of a specific antagonist, the 9-mer peptide F991. Acute allergic responses induced by IgG or IgE are not inhibited by F991.^{12,14} Ig-fLCs are present in the serum, and their production is augmented under inflammatory conditions, including allergic asthma, as well as some autoimmune diseases.^{12,15,16} For example, in patients with multiple sclerosis, free κ light chains correlate with disability prognosis,¹⁷ and in patients with rheumatoid arthritis, there is a significant correlation between κ and λ fLCs and the disease activity score.¹⁸

In this study we demonstrate that circulatory Ig-fLC concentrations are increased after oral sensitization with casein. Moreover, acute allergic skin reactions to casein are inhibited by F991, which indicates that Ig-fLCs play a pivotal role in the allergic sensitization. Clinical data indicate that Ig-fLC concentrations are increased in children with CMA and in children at risk for allergy with mild atopic dermatitis (AD).

METHODS**Chemicals**

Casein and whey were obtained from DMV International (Veghel, The Netherlands). Cholera toxin was purchased from Quadrtech Diagnostics (Epsom, United Kingdom). PBS was from Cambrex Bio Science (Verviers, Belgium). Biotin-labeled rat anti-mouse IgE and IgG₁ were from BD Biosciences (Alphen aan den Rijn, The Netherlands). The Ig-fLC antagonist F991 (AHWSGHCCCL) was synthesized by Fmoc chemistry (Ansynth, Roosendaal, The Netherlands). All other chemicals were obtained from Sigma-Aldrich-Chemie (Zwijndrecht, The Netherlands).

Oral sensitization and challenge of mice

Three- to 5-week-old specific pathogen-free female C3H/HeOJ mice ($n = 6$ per group) were purchased from Charles River Laboratories (Maastricht, The Netherlands), maintained on CMP-free mouse chow (Special Diets Services, Witham, Essex, United Kingdom), and housed in the animal facility at Utrecht University. Animal care and use were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of the Utrecht University. Mice were sensitized intragastrically with 0.5 mL of homogenized casein or whey (40 mg/mL PBS) with cholera toxin (20 μ g/mL PBS) as an adjuvant by using a blunt needle. Control mice received cholera toxin alone or PBS. Mice were boosted weekly for a period of 5 weeks. One week after the last sensitization, an intradermal ear challenge was performed. After 24 hours, blood samples were collected and centrifuged (15 minutes at 16,000g). Sera were stored at -70°C . Mice were killed by means of cervical dislocation. Cells of spleen and mesenteric lymph nodes of sensitized mice were collected and cultured in serum free RPMI (1×10^6 cells/mL) for 24 hours.¹⁴ Supernatants were collected to measure immunoglobulin levels, Ig-fLC levels, or both.

Measurement of specific serum immunoglobulins

Levels of casein- or whey-specific IgE and IgG₁ were determined in serum by means of ELISA. Microtiter plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 μ L of whey or casein (20 μ g/mL) in coating buffer (carbonate-bicarbonate buffer, 0.05 mol/L, pH 9.6) for 18 hours at 4°C . Plates were washed and blocked for 1 hour with 5% BSA. Serum samples were applied in several dilutions and incubated for 2 hours at room temperature. Plates were washed and incubated with biotin-labeled rat anti-mouse IgE or IgG₁ (1 μ g/mL) for 1 hour at room temperature and washed. The plates were incubated with streptavidin-horseradish peroxidase for 1 hour, washed, and developed with *o*-phenyldiamine. After 5 minutes, the reaction was stopped with 4 mol/L H_2SO_4 , and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, Calif). Results were expressed as arbitrary units (AU), with pooled sera from casein and whey alum intraperitoneally immunized mice used as positive reference sera to make the titration curve as an internal standard.

Immunoblotting Ig-fLCs

Before use, serum samples were precipitated to deplete high amounts of albumin by using trichloroacetic acid/acetone, as previously described.¹⁹ Samples were fractionated by means of SDS-PAGE under nonreducing conditions, electroblotted to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Veenendaal, The Netherlands) overnight, and probed with anti-mouse κ horseradish peroxidase antibody (Southern Biotech, Birmingham, Ala). Immunoreactive bands were visualized by using enhanced chemiluminescence. Optical density (OD) of the immunoreactive bands was quantified with a calibrated densitometer (Bio-Rad Laboratories).

Acute allergic skin reaction

The allergen-specific skin reaction was measured after injection of the specific protein in the ear pinnae. Nonsensitized mice were injected intradermally in the left ear with 20 μ L of casein (0.5 mg/mL in PBS) and in the right ear with whey (0.5 mg/mL in PBS) to determine nonspecific skin reactions. The casein- or whey-sensitized mice were injected in both ears with casein or whey, respectively, whereas sham-sensitized mice were injected with casein in the left ear and whey in the right ear. Ear thickness of mice was measured in duplicate by using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands) at time 0 and 1 hour after challenge. Ear swelling is expressed in micrometers.

Passive transfer with spleen supernatants

Spleen supernatants were concentrated by using Vivaspinn 20 spin filters (Millipore, Temecula, Calif). Concentrated fractions were 0.2 μ m filtered and transferred intravenously (100 μ L, approximately 400 μ g of protein/mL) in naive recipient mice. Sham mice received PBS as a control. After half an hour, skin reaction tests were performed, as described above.

Antagonist studies

In the orally sensitized mice, as well as in mice passively transferred with spleen supernatants of sensitized mice, F991, an Ig-fLC inhibitor,^{14,20} or PBS as a control was administered intravenously (100 μ L, 0.2 mg/mL F991) to determine whether the ear swelling was Ig-fLC dependent. Half an hour after F991 administration, the ear swelling test was performed as described above.

Subjects with CMA

Infants aged 12 months or less and suspected of having CMA were recruited from the Baby Health Clinics in the region of Amsterdam, The Netherlands. Baby Health Clinics are involved in the prevention and early detection of diseases in infancy and early childhood, in growth and development, and in providing nutritional advice. Ninety-eight percent of the newborns are regularly controlled in this manner. Baby Health Clinic physicians are often the first to be confronted with symptoms of CMA. Infants suspected of CMA

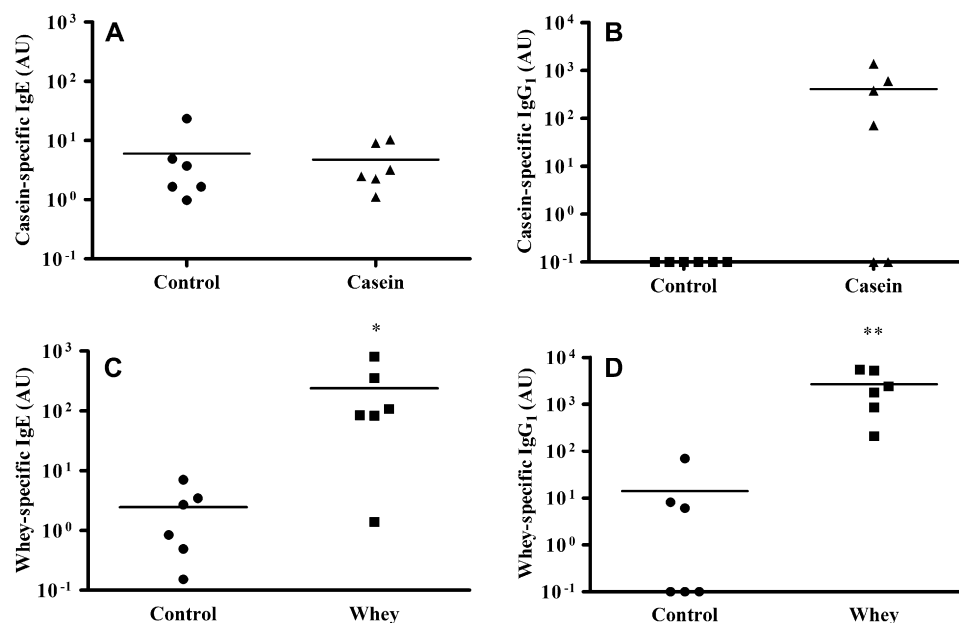


FIG 1. Specific serum immunoglobulin levels in casein- and whey-sensitized mice. In the casein-sensitized mice no increase in casein-specific IgE levels was found (A), but the IgG₁ level was increased in 4 of 6 animals (B). Whey sensitization induced levels of specific IgE (C) and IgG₁ (D). **P* < .05 and ***P* < .01 (*n* = 6).

according to standardized criteria were referred to the Emma Children's Hospital Academic Medical Center, Amsterdam, The Netherlands, for evaluation of symptoms and diagnostic workup. The following symptoms related to ingestion of CMPs were considered suspect for CMA: skin symptoms (AD, urticaria, angioedema, and erythema), gastrointestinal symptoms (colic, vomiting, and diarrhea), respiratory symptoms (rhinitis, cough, wheeze, and dyspnea), and general symptoms (inconsolable crying, refusing food, and failure to thrive). AD was diagnosed according to criteria from Hanifin and Rajka.²¹ The extent and intensity of AD was estimated by using the objective SCORAD index.^{22,23}

Diagnostic procedure. CMA was diagnosed according to a strict protocol based on elimination of CMPs from the diet, a DBPCFC with CMPs, and re-elimination of CMPs from the infant's diet according to international recommendations.²⁴

The elimination of CMPs from the infant's diet was performed with a minimum duration of 1 week and a maximum duration of 6 weeks. An infant formula based on a mixture of free amino acids (Neocate; SHS International, Liverpool, United Kingdom) was provided to bottle-fed infants to eliminate CMPs from each infant's diet. CMPs were excluded from the diets of mothers with breast-fed infants by means of strict elimination of CMP, soy, and hen's egg. If symptoms suspicious of CMA did not improve during the elimination phase, the infant was excluded from the study. Otherwise, if symptoms disappeared or improved significantly, a DBPCFC with CMP was conducted.

The active and placebo arms of the DBPCFC were conducted in a random order on 2 separate days with an interval of 2 to 7 days. A randomization database (www.randomization.com) was used to assess the order of the active and placebo arms. During active challenges, skimmed milk powder (Institute of Food Research, Norwich, United Kingdom) dissolved in Neocate was given in the following subsequent doses: 0.2 mL (0.003 mg), 2 mL (0.03 mg), 20 mL (0.3 mg), 0.2 mL (3 mg), 2 mL (30 mg), 6 mL (100 mg), 20 mL (300 mg), 60 mL (1,000 mg), and 200 mL (3,000 mg). Placebo challenges were performed by administering the same volume of 100% Neocate. The time interval between each dose was 20 minutes. The challenge was stopped if clinical symptoms were observed or the highest dose was reached. The infants were observed for 2 hours after each challenge. A challenge result was defined as positive if objective clinical reactions were observed. The objective SCORAD index was used to assess the severity of AD before and 2 hours after each challenge. Clinical reactions after discontinuation or within 2 hours after the highest dose were defined as early reactions, and those thereafter were defined as

late reactions. A standardized sheet was used for documenting administered doses and clinical reactions.

Elimination of CMP from the infant's diet was continued at the days between the active and placebo arms of the DBPCFC until 1 week (re-elimination) after the last challenge. One week after the last challenge, the code was broken, and the outcome of the DBPCFC was assessed. CMA was diagnosed based on a positive DBPCFC result. Subjects with a negative DBPCFC result and cow's milk-specific IgE levels in the normal range were included as nonallergic control subjects.

Serum immunoglobulin and plasma Ig-fLC analysis.

Peripheral blood samples were collected before challenge on the first day of the DBPCFC. Cow's milk-specific IgE levels were determined by using the CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). In addition, total levels of Ig-fLC κ and λ were determined, as described previously.^{12,25}

Ethical consideration. The study (MEC 05/254) was approved by the medical ethical committee of the Academic Medical Center, Amsterdam, The Netherlands. Parental informed consent was obtained for all subjects.

Infants at high risk for allergy: Serum Ig-fLC κ and λ levels

Term-born infants with a parental history of atopic eczema, allergic rhinitis, or asthma in either the mother or father were eligible for a prebiotic intervention study, which was described previously.^{26,27} The study protocol was approved by the Ethical Committee of the Macedonio Melloni Maternity Hospital. In a subpopulation plasma immunoglobulin levels were measured as described by Van Hoffen et al (CMP: IgE and IgG₁; total: IgE, IgG₁, and IgG₂).²⁷ In total, 25 infants had mild symptoms of AD (SCORAD, 5.2–24.7), and 49 infants did not have AD at the age of 6 months. For this study, total levels of Ig-fLC κ and λ were determined in plasma, as described previously.^{12,25}

Statistical analysis

Ear-swelling data and murine Ig-fLC data were analyzed by using 1-way ANOVA, and the *post hoc* Dunnett test and all other serologic data were analyzed by using the 2-tailed Mann-Whitney test. Statistical analyses were conducted with GraphPad Prism software (version 4.03; GraphPad Software, Inc, La Jolla, Calif; **P* < .05, ***P* < .01, and ****P* < .001). Murine data are represented as means $\pm$ SEMs, whereas clinical data are represented as medians.

RESULTS

Serum specific immunoglobulin levels

Serum levels of casein-specific IgE were not increased in mice orally sensitized with casein when compared with those seen in control mice (4.7 ± 1.6 vs 6.0 ± 3.5 AU, respectively; Fig 1, A). In contrast, casein-specific IgG₁ levels were enhanced (404.8 ± 217.5 vs 0 ± 0 AU, respectively), although this was not significant because of 2 nonresponders (Fig 1, B). In whey-sensitized mice both specific IgE (239.4 ± 123.6 AU, $P < .05$) and IgG₁ ($2,671 \pm 910$ AU, $P < .01$) levels were significantly increased compared with those seen in sham-sensitized mice (2.5 ± 1.1 and 14.0 ± 11.2 AU, respectively; Fig 1, C and D).

Ig-fLC levels in sensitized mice

Next it was examined whether sensitized mice had enhanced total levels of Ig-fLCs. Sera of nonsensitized, casein-sensitized, and whey-sensitized mice were analyzed by immunoblotting for the presence of Ig-fLC κ . The concentrations of Ig-fLC κ in casein-sensitized mice were significantly increased compared with those seen in nonsensitized and whey-sensitized animals ($P < .05$; nonsensitized, 66.6 ± 5.5 ; whey sensitized, 64.2 ± 8.7 ; and casein sensitized, 92.3 ± 9.5 OD/mm²; Fig 2, A).

To analyze production of Ig-fLCs in local lymph nodes, cells from mesenteric lymph nodes ($n = 6$ pooled) were cultured for 24 hours, and supernatants were analyzed for Ig-fLC κ . Samples fractionated by means of SDS-PAGE under nonreducing conditions showed complete immunoglobulins (about 180–200 kD), Ig-fLC dimers (about 45 kD), and Ig-fLC monomers (20 kD). The mesenteric lymph nodes of casein-sensitized mice produced higher levels of Ig-fLCs compared with levels seen in the control and whey-sensitized animals (Fig 2, B).

Ig-fLC inhibitor F991 blocks the acute allergic skin reaction in casein-sensitized mice

The effect of the antagonist F991 on allergen-induced ear swelling was tested to analyze whether the increase in Ig-fLC levels in casein-sensitized animals was functionally relevant regarding the allergic reaction. F991 greatly reduced the skin reaction in the casein-sensitized mice (147.4 ± 16.0 μ m without and 83.3 ± 8.3 μ m with F991, $P < .05$; Fig 3, A). As expected, the Ig-fLC antagonist did not affect ear swelling in whey-sensitized mice (145.0 ± 9.66 and 148.5 ± 13.9 μ m, respectively; Fig 3, B). Also, nonspecific ear swelling (irritant reaction) in control mice was not affected by F991. Casein did not provoke ear swelling in mice with whey allergy and *vice versa* (data not shown).

Ig-fLCs responsible for passive transfer of allergic sensitization to casein

In previous experiments it was shown that local lymphoid organs from casein-sensitized mice produced significantly more Ig-fLCs compared with those of sham- and whey-sensitized animals. To investigate the involvement of Ig-fLCs as a humoral factor in the induction of the acute allergic skin reaction, naive recipient mice were injected intravenously with concentrated spleen supernatants (or PBS as a control) obtained from allergic mice in the presence or absence of F991, as previously described.¹⁴ Subsequently, the cutaneous swelling was measured at 1 hour after intradermal allergen challenge. For recipient mice

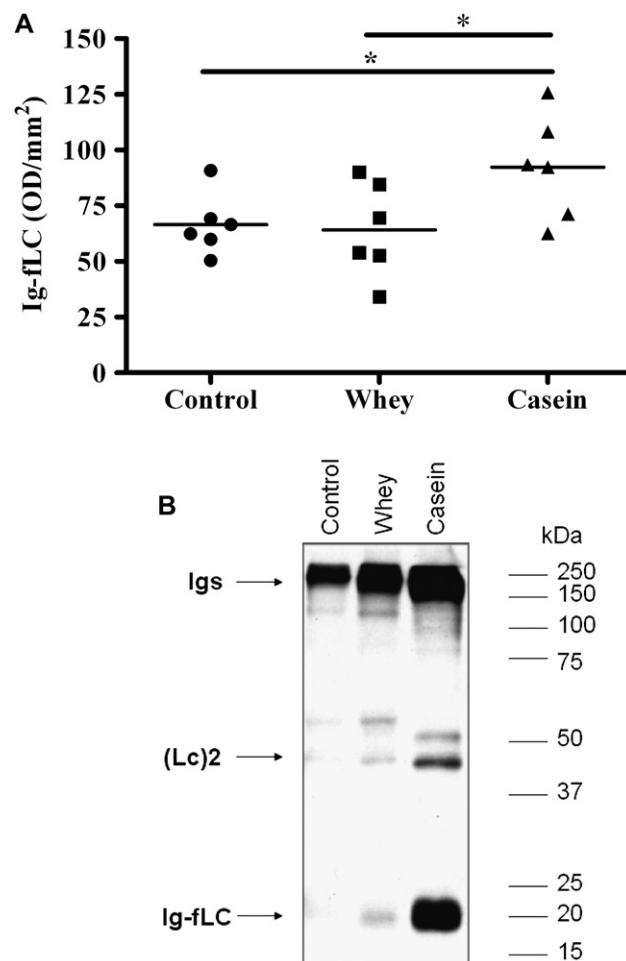


FIG 2. Densitometric analysis of κ Ig-fLC in serum and supernatants from local mesenteric lymph nodes revealed Ig-fLC levels to be increased. **A**, Analysis of κ Ig-fLC in sera from individual mice. In casein-sensitized mice Ig-fLC levels were significantly increased in comparison with those seen in control- or whey-sensitized mice. **B**, Representative Western blot of Ig-fLC levels in supernatants of mesenteric lymph nodes of control-, casein-, and whey-sensitized mice. Ig-fLC, Monomers; Igs, total immunoglobulins (IgA, IgD, IgE, IgG, and IgM); (Lc)2, Ig-fLC dimers. * $P < .05$ ($n = 6$).

receiving spleen supernatants of casein-sensitized mice, the allergen challenge induced significant ear swelling (110.9 ± 10.2 μ m). F991 inhibited the casein-induced ear swelling highly significantly (57.1 ± 5.7 μ m, $P < .001$, Fig 4). The Ig-fLC antagonist did not affect the ear swelling of recipient mice receiving spleen supernatants of whey-sensitized mice (105.9 ± 6.6 and 105.0 ± 5.1 μ m), confirming that Ig-fLCs did not play a significant role in induction of the acute allergic skin reaction against whey protein. Casein did not provoke ear swelling in whey spleen supernatant-transferred mice and *vice versa* (data not shown).

Immunoglobulin and Ig-fLC levels in infants with CMA

Sixteen infants (age, 2.5–8.9 months; median age, 5.4 months) with a positive DBPCFC result and 17 infants (age, 3.1–7.3 months; median age, 4.0 months) with a negative DBPCFC result and cow's milk-specific IgE levels in the normal range for age were included in this study. Both Ig-fLC κ (median, 5.3 [interquartile range {IQR}, 3.1] vs 10.1 [IQR, 5.2]; $P = .0042$;

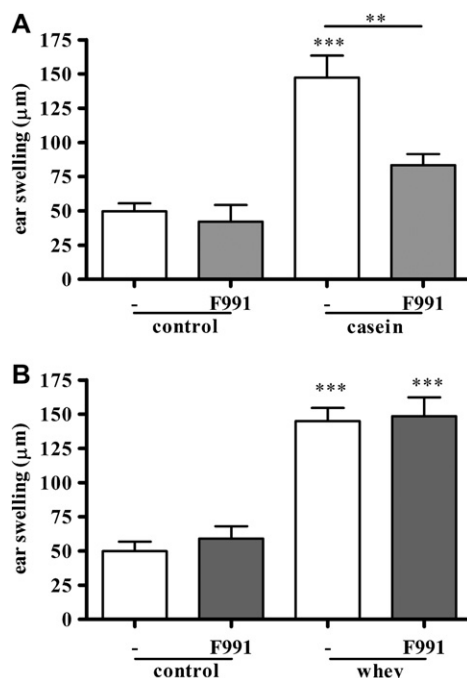


FIG 3. Effect of F991 on the induction of an acute allergen-specific skin reaction in casein-sensitized (A) and whey-sensitized (B) mice. F991 decreased casein-induced ear swelling significantly, but whey-induced ear swelling was unaffected. Ear swelling (in micrometers) is calculated as the increase in ear thickness induced by the corresponding antigen at 1 hour after challenge. $**P < .01$ and $***P < .001$ ($n = 6$).

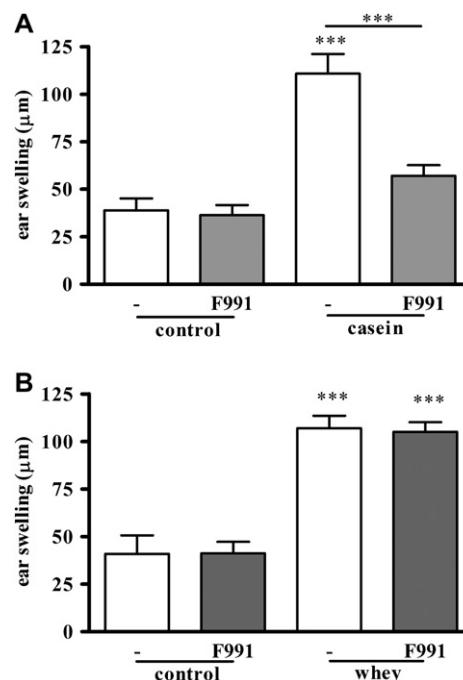


FIG 4. Effect of F991 on the induction of an acute allergic skin reaction in naive mice injected intravenously with spleen supernatants or PBS as a control. Passive transfer with spleen supernatants of mice with casein (A) and whey (B) allergy resulted in an acute allergic skin reaction on intradermal allergen challenge. Only in casein recipients was the acute allergic skin reaction abrogated by F991. $***P < .001$ ($n = 6$).

Fig 5, A) and Ig-fLC λ (median, 6.8 [IQR, 5.7] vs 8.6 [IQR, 6.0]; $P = .0222$; Fig 5, B) concentrations were significantly enhanced in children with CMA. Ig-fLC κ or λ levels did not differ in the subgroups of infants with CMA with detectable and undetectable IgE levels (data not shown).

Immunoglobulin and Ig-fLC levels in infants at high risk for allergy

Immunoglobulin and Ig-fLC levels were also measured in plasma from children at risk of allergic disease from a double-blind, randomized, placebo-controlled prebiotic intervention study to extend the clinical relevance of the current findings.^{26,27} These infants were 6 months of age and had AD (SCORAD between 5.2 and 24.7) or remained negative for AD (SCORAD $-$). No differences were found between the 2 groups for total levels of IgE, IgG₁, and IgG₂ and levels of cow's milk-specific IgE and IgG₁ (Table I).

In support to our findings in the preclinical and clinical studies, Ig-fLC κ levels were found to be increased in infants with AD ($n = 25$) when compared with those in infants without AD ($n = 49$, $P = .0075$). Ig-fLC λ levels showed a similar tendency ($P = .14$).

DISCUSSION

In previous studies it was shown that the allergic skin response in mice sensitized with casein or whey, both major allergens responsible for CMA, is substantially different.^{10,11,28} Whey-sensitized mice show a classical type I allergic skin reaction combined with high levels of specific IgE. However, in casein-sensitized mice acute allergic responses were elicited in the

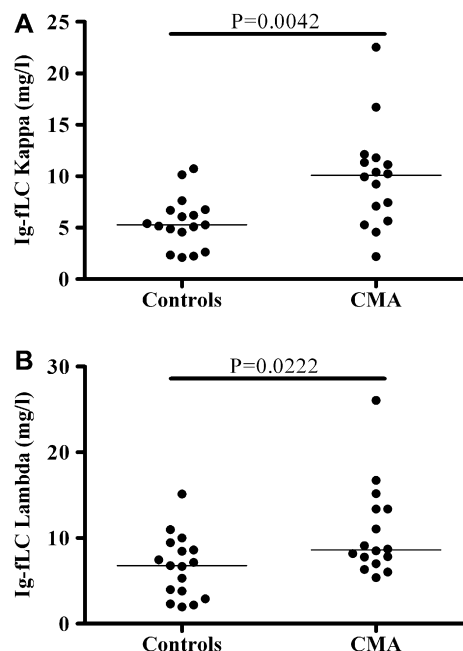


FIG 5. Plasma Ig-fLC levels in nonallergic control subjects ($n = 17$) and infants with CMA ($n = 17$). Both κ Ig-fLC levels ($P = .0183$; A) and λ Ig-fLC levels ($P = .0328$; B) were enhanced in infants with CMA.

absence of casein-specific IgE. Here new data provide evidence that casein sensitization of mice results in the induction of an Ig-fLC-dependent acute hypersensitivity response. In addition, plasma Ig-fLC concentrations were found to be enhanced in

TABLE I. Immunoglobulin levels in infants at high risk for allergy divided into healthy (SCORAD –) and AD (SCORAD +) groups

		AD		
		SCORAD –	SCORAD +	
		n = 49	n = 25	
		Median (IQR)	Median (IQR)	
Serum antibody	Isotype	Median (IQR)	Median (IQR)	P value
Total	IgE (kU/L)	4.50 (9)	6.00 (22)	.30
	IgG ₁ (g/L)	2.69 (1.9)	2.95 (2.5)	.15
	IgG ₂ (g/L)	0.78 (0.4)	0.91 (0.6)	.41
	Ig-fLC κ (μ g/mL)	6.1 (4.0)	7.4 (3.0)	.0075
	Ig-fLC λ (μ g/mL)	6.1 (4.1)	7.7 (5.6)	.14
CMP specific	IgE (ng/mL)	1.95 (3.1)	2.90 (3.2)	.10
	IgG ₁ (AU/mL)	1.50 (6.5)	3.25 (7.7)	.27

IQR, Interquartile range.

patients with CMA, as well as in patients with AD, implying a possible contribution of Ig-fLCs to the pathophysiology of clinical allergic disease.

Food allergy is thought to be mediated by IgE- and non-IgE-dependent mechanisms. IgE-mediated food allergy is a classical type I allergy, with symptoms occurring within a few hours. Non-IgE-mediated food allergy usually is defined as a delayed-type hypersensitivity with enhanced T_H1 activity, and the symptoms are noticeable after 1 or more days while cellular mechanisms are involved.²⁹⁻³³ However, of interest is a subgroup of patients who have symptoms of an immediate-type hypersensitivity response while lacking detectable levels of IgE.^{3,5,6} In contrast to the murine model, in human subjects caseins can elicit strong IgE responses.³ The mice were sensitized for either casein or whey, whereas in infants hypersensitivity is raised against whey and casein derived from whole cow's milk. Direct comparison between mice and human subjects with regard to the sensitizing capacities for casein and whey are therefore difficult to perform. However, the concept of Ig-fLCs being involved in the pathophysiology of human disease has been described for inflammatory, as well as allergic, asthma.^{12,14,17,18,34,35}

Several lines of evidence suggest the critical involvement of Ig-fLCs in the induction of casein hypersensitivity in mice. First, casein sensitization results in increased levels of Ig-fLCs in serum, whereas local lymph nodes of casein-sensitized mice show enhanced production of Ig-fLCs *ex vivo*.

Second, inhibition of Ig-fLC-induced hypersensitivity with F991 prevented the acute allergic skin response in casein-sensitized mice. A minor portion of ear swelling seen in casein-sensitized mice was not affected by F991, which could suggest a residual role for IgG₁ or IgE. In several studies it has been shown that F991 is specific for Ig-fLCs and does not affect IgE- or IgG-mediated skin responses. Furthermore, F991 prevents local inflammation in different hypersensitivity models.^{12,14,20,36}

Third, allergic sensitivity to casein could be transferred to naive animals by means of intravenous injection of culture supernatants from splenocytes of casein-sensitized mice and was found to be dependent on Ig-fLCs. Together, these data indicate a prominent role for Ig-fLCs in the induction of casein allergy in mice. In another study (data not shown) mice were sensitized with both casein and whey, and mice were subsequently challenged intradermally with either casein or whey. Both casein and whey challenge provoked an acute allergic skin response, whereas casein-specific IgE levels remained less than the detection level.

Other studies with murine models of CMA indicate various and sometimes conflicting results. Oral sensitization with α -casein resulted in an increase in α -casein-specific IgE and IgG₁ titers in C3H mice.³⁷ On the other hand, Lara-Villoslada et al³⁸ only detected whey- and casein-specific IgG₁, whereas IgE was not detectable in a CMA model induced by simultaneous oral sensitization with both casein and whey. The differences in route of sensitization (intraperitoneal vs oral), murine strains, and variation in allergen fractions (eg, α -casein vs complete casein fraction or combined whey and casein) might explain the contrasting outcome in these and the present studies. The fundamental different mechanisms of allergy observed for casein and whey might be due to the differences in digestive speed and the dietary, chemical, physical, and physiological features of the proteins.³⁹⁻⁴² Therefore these differences between the proteins influence not only the intestinal physiology but also likely the response of the immune system, which is a topic for further studies.

In previous studies it has been shown that Ig-fLCs can mediate immediate hypersensitivity-like responses. Mast cells are the primary target cells for Ig-fLCs, and subsequent cross-linking of cell surface-bound Ig-fLCs by cognate antigen results in mast cell activation and the induction of a local inflammatory response.¹⁴ Polyclonal Ig-fLC levels are enhanced in chronic inflammatory diseases, such as inflammatory bowel disease,⁴³ rheumatoid arthritis,¹⁶ Sjögren syndrome,¹⁸ multiple sclerosis,^{17,44} and systemic lupus erythematosus.³⁴ Also in allergic and nonallergic asthma, Ig-fLC levels are increased when compared with those seen in healthy control subjects.¹² It is estimated that in one third of the children with AD, the skin symptoms are triggered by sensitization to food.^{45,46} Notably, children with severe AD have higher levels of λ and κ light chains in sera.⁴⁷ Furthermore, in patients with non-IgE-mediated rhinitis, both λ and κ Ig-fLC levels are found to be enhanced in nasal secretions.³⁵ The present study confirms that increased Ig-fLC levels might be associated with development of CMA. In sera from patients with DBPCFC-confirmed CMA, both κ and λ Ig-fLC concentrations were significantly enhanced compared with those seen in control subjects. Ig-fLC concentrations did not differ between infants with IgE- and non-IgE-mediated CMA, indicating that Ig-fLCs might contribute to the allergic symptoms in patients with non-IgE-mediated CMA. A larger cohort of patients with non-IgE-mediated CMA is required to elucidate this. Plasma from infants at high risk for allergy who had mild AD were investigated as well to extend the evidence of a possible role of Ig-fLCs in

allergic disease developed during early infancy. Neither enhanced CMP-specific serum IgE and IgG₁ levels nor enhanced total IgE, IgG₁, and IgG₂ levels were observed in these children. However, serum levels of κ Ig-fLC were increased significantly, and λ Ig-fLC levels showed a similar increment. These clinical data suggest that measurement of total and, ultimately, specific levels of Ig-fLCs might have relevance in the clinical diagnosis of CMA and AD.

In summary, sensitization with CMPs can lead to both IgE-dependent and IgE-independent allergic hypersensitivity responses. In this study evidence is provided indicating that the IgE-independent allergic response to casein is mediated at least in part by specific Ig-fLCs. An increase in Ig-fLC levels might be of relevance in patients with CMA and AD with or without noticeable involvement of IgE. Further research is warranted to determine the clinical significance of assaying Ig-fLCs for the diagnosis of allergic diseases.

Clinical implications: IgE-independent casein-specific acute allergic reactions in mice were found to be caused, at least in part, by Ig-fLCs. Ig-fLC-mediated hypersensitivity reactions might occur in clinical allergy.

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