



Characteristics of patients suffering from cow milk allergy

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ABSTRACT

The most frequent symptoms among the manifestations of cow milk allergy (CMA) are gastrointestinal. CMA pathogenesis involves immunological mechanisms with participation of immunocompetent cells, production of immunoglobulin E (IgE) and immunoglobulin G (IgG). We aim to determine whether cow milk-specific IgE antibodies coexist with cow milk-specific IgG antibodies in CMA patients with diarrhea symptom, and if there is any relationship between both antibody types. 65 CMA patients (average age of 17 years, ranging from 2 to 74 years), all of who had diarrhea symptom of CMA, were enrolled in this study. The total cow IgE and IgG subclass in serum were measured by electrochemiluminescence immunoassay and rate immune scatter turbidimetry, respectively. And also the cow milk-specific IgE was determined by enzyme-linked immunosorbent assay. The number of eosinophils in serum was calculated by Sysmex XE-2100 Hematology Analyzer. Our data showed that both cow milk-specific IgG and IgE levels were significantly elevated in CMA patients compared to those of age-matched control subjects. Out of the 65 CMA patients, 40 showed elevated cow milk-specific IgE antibody level, among which, 28 cases presented highly sensitive reaction to cow milk-specific IgG, along with each six of moderate and mild sensitive reaction to cow milk-specific IgG; while 20 showed elevated total IgG levels. The IgG3 positive rate was 16.9%, which was the highest. A moderate correlation between cow milk-specific IgE and cow milk-specific IgG was found in the CMA patients ($r=0.415$, $P=0.001$). The results indicated that cow milk-specific IgE antibodies could coexist with cow milk-specific IgG antibodies in patients suffering from CMA. The aberrant changes in the concentration of cow milk-specific IgE antibodies were associated with cow milk-specific IgG antibodies.

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1. Introduction

Human milk is the optimal food for human infants, but cow milk usually substitutes for human milk when breast-feeding is not available. This substitution can lead to nutritional and immunological problems, such as cow milk protein allergy. The milk proteins act as antigens in the body, and promote the production of milk-specific antibodies, which consequently lead to the inflammation response in the corresponding tissues. Finally signs and symptoms will appear in the body [1]. Milk hypersensitivity in early childhood is mostly mediated

by milk protein immunoglobulin E (IgE) [2] and dependent on the activation of mast cells in specific tissues, including the skin, respiratory tract, gastrointestinal, mucosal, and cardiovascular system [3–5,12]. Milk allergy has also been involved in type I allergies. The impact of other types of immune reactions to cow milk is presently controversial, such as the association of antibodies of immunoglobulin G (IgG) and immunoglobulin A (IgA) isotypes with cow milk-induced adverse gastrointestinal symptoms in adults [3,4]. A new scientific publication by Jönsson et al. [5] proved previous scientific findings [6,7] that IgG could also be implicated in anaphylaxis. Another study showed similar levels of IgA and IgG antibodies against cow milk proteins in healthy individuals and in CMA patients [8]. IgG-mediated CMA can take several days before its appearance. Immune complexes are then formed, causing partial or systemic type III or type IV allergies [9–12].

The objective of current research is to study whether cow milk-specific IgE antibodies coexist with cow milk-specific IgG antibodies in CMA patients with diarrhea symptom, and if there is any relationship between both antibody types.

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2. Materials and methods

2.1. Materials

In all, 65 CMA patients (average age of 17 years, ranging from 2 to 74 years, including 44 males and 21 females), who sought medical advice for the reason of diarrhea from November 2009 to September 2010, were screened at the Department of Digestive System of Xiamen Zhongshan Hospital. The clinical diagnosis of cow milk allergy was based on World Allergy Organization Diagnosis and Rationale for Action against Cow Milk Allergy guidelines [13,14]. The detailed characteristics of the investigated study groups were shown in Table 3. 45 healthy subjects (average age of 18 years, ranging from three to 70 years, including 24 males and 21 females) were also recruited. For infant's selection, all those breast-fed infants were excluded, and only formula cow milk-fed ones were involved. 6 mL blood samples were draw from all subjects followed by the completion of questionnaires on gastrointestinal symptoms and dairy consumption. This research was subjected to the approval of local ethics committee, and full written informed consent was obtained from the subjects.

2.2. Laboratory test

2.2.1. Measurement of milk-specific IgG antibody in serum

Blood samples were allowed to stand for 20–30 min before centrifugation at 3000 RPM for 15 min. The serum was aspirated and frozen at -20° for subsequent analysis. Samples were processed in the central laboratory (Xiamen Zhongshan Hospital, China) using a commercially available enzyme-linked immunosorbent KIT (ELISA, Biomerica, Inc., Newport Beach, CA, USA, Kit number: 7145-HA). All manipulations were according to the instructions of the ELISA Kit, and the following are the brief procedures.

Prepare 50, 100, 200 and 400 U/mL of milk IgG calibrator and add into the microplate; set up blanks and positive controls. This was the calibration curve to be used in the assay. Dilute serum samples by 100 fold with serum dilute reagents. Then add 100 μ L of samples into the microwells precoated with milk antigens. Cover plate with plate cover and incubate for 60 min at room temperature. Thoroughly decant any liquid from wells and wash wells 4 times. Add 100 μ L of milk IgG-HRP into each well; incubate for 30 min at room temperature. Thoroughly decant any liquid from wells and wash wells 4 times. Add 100 μ L of working substrate mix (TMB and H_2O_2) into each well. Incubate for 10 min at room temperature in the dark. Add 50 μ L of 1 N sulfuric acid per well to stop the reaction. Read the absorbance of each well at 450 nm using a BIO-RAD 550 ELISA plate reader (Bio-Rad Laboratories, Inc, USA). The IgG concentration against milk antigen was expressed as units per milliliter. And the following Table 1 defined the sensitive ranks according to IgG concentration.

2.2.2. Measurement of milk-specific IgE antibody in serum

Milk-specific IgE antibody in serum was measured using an ELISA kit (DR. FOOKE Laboratorien GmbH, Germany, Kit number: K980818). And the following Table 2 defined the sensitive ranks according to IgE concentration.

Table 1

The sensitive ranks according to IgG concentration.

Cow milk-specific IgG concentration (U/mL)	Rank
<50	Negative
50–100	Mild
100–200	Moderate
>200	High

Table 2

The sensitive ranks according to IgE concentration.

Cow milk-specific IgE concentration (U/mL)	Rank
<0.35	Negative
0.35–0.70	Low
0.70–3.50	Moderate
>3.50	High

2.2.3. Measurements of total serum IgG, IgG subclass antibody, total IgE and eosinophils

Total IgG and IgG subclass were measured using a full automation specific protein analyzer (Siemens, Germany, Total IgG, IgG1, IgG2, IgG3, IgG4 kit numbers 153063, 090145, 090249, 086145, 086046). The total IgE concentration was measured using an electrochemiluminescence immunoassay analyzer (Roche Modular Analytics E170; Roche Diagnostics, Mannheim, Germany, Kit number: 162012). The number of eosinophils was calculated with a Sysmex XE-2100 Hematology Analyzer (Sysmex, Kobe, Japan). All tests were performed following the manufacturer's specifications.

2.3. Statistical analysis

All statistical analysis was conducted using SPSS for Windows version 13. The data were expressed as mean $\pm$ standard deviation. Means of two independent groups were compared with an unpaired Student's t-test. Chi-square tests were employed to determine the significant differences across groups. The correlations between cow milk-specific IgG and cow milk-specific IgE were assessed using Pearson's correlation test. $P < 0.05$ (2-tailed) was considered significant.

3. Results

3.1. Cow milk-specific IgG, cow milk-specific IgE and total IgE levels in CMA patients

Serum from CMA patients and controls were analyzed by ELISA and electrochemiluminescence immunoassay regarding their IgG and IgE reactivity to cow milk. Cow milk-specific IgG and IgE levels were significantly higher in CMA patients with diarrhea predominant symptom than those in healthy subjects (Table 4).

3.2. Serum IgG level

Serum from 65 CMA patients suffering from diarrhea were subjected to ELISA for measurement of cow milk-specific IgG. The cow milk-specific IgG concentration was 267.8 ± 195.1 U/mL which was positive. Among the 65 patients, 15 (23.1%) showed mildly sensitive reactivity, 17 (26.2%) were moderately sensitive reactivity, and 33 (50.8%) were highly sensitive reactivity. The concentrations of cow

Table 3

Characterization of study groups.

Number of patients with cow's milk allergy in diagnosis	65
Average age ^a	17 (2–74)
Males/females	44/21
Mean introduction time of cow's milk protein ^b	1–7
Presenting symptoms	
Gastrointestinal	65
Number of controls	45
Average age ^a	18 (3–70)
Males/females	24/21
Mean introduction time of cow's milk protein	–

^a Years. Results are expressed as mean value (scope).

^b Days. Results are expressed as range.

Table 4
Cow milk-specific IgG, cow milk-specific IgE and total IgE levels.

	Cases (n)	Specific IgG to CM (range, U/mL), positive rate (%)	Specific IgE to CM (range, U/mL), positive rate (%)	Total IgE (range, ng/mL), positive rate (%)
CMA patients	65	52.6–779.0 (100%)	0–47.76 (40.0%)	9.6–5395.0 (55.4%)
Healthy subjects	45	0–60.0 (4.4%)	0–0.41 (2.2%)	5.8–531.2 (2.2%)
Fisher exact probability test <i>P</i> value		0.000	0.000	0.000

CM: cow milk; CMA: cow milk allergy.

milk-specific IgG in these groups were 71.1 ± 14.8 , 139.2 ± 26.9 , and 423.5 ± 154.0 U/mL, respectively.

Total serum IgG and IgG subclasses in the 65 CMA patients were determined at the same time. 20 patients were total IgG-positive. The IgG3 positivity rate (16.9%) was the highest, followed by IgG2 (10.8%), IgG1 (3.1%), and IgG4 (1.5%).

3.3. Serum IgE and eosinophil levels

Cow milk-specific IgE was determined with ELISA. Out of the 65 CMA patients with diarrhea symptom, 40 (61.5%) patients showed elevated levels of cow milk-specific IgE. There was no significant difference in cow milk-specific IgE concentration between the male and female patients (data not shown). Cow milk-specific IgE levels varied with ages. The cow milk-specific IgE concentration of younger patients (age ≤ 18 years old, $5.842 \text{ U/mL} \pm 12.29 \text{ U/mL}$) was significantly higher than those of adult patients (age > 18 years old, $0.641 \text{ U/mL} \pm 1.603 \text{ U/mL}$) which was increased by 9.1-fold in younger patients (Table 5). 40 CMA patients had immune response to cow milk-specific IgE, among which, 13 (32.5%) had low reactions to cow milk-specific IgE, with concentration of $0.5446 \pm 0.0835 \text{ U/mL}$; 17 (42.5%) had moderate reactions to cow milk-specific IgE, with concentration of $1.470 \pm 0.840 \text{ U/mL}$; and 10 (25.0%) had high reactions to cow milk-specific IgE, with concentration of $23.41 \pm 16.74 \text{ U/mL}$.

Among the 65 CMA patients suffering from diarrhea symptom, total serum IgE value in 36 (55.4%) was elevated and the absolute eosinophil number in 10 (15.4%) was lifted. The levels of both total serum IgE antibodies and cow milk-specific IgE antibodies simultaneously went up in 23 patients (57.5%). Evidently, cow milk-specific IgE in CMA patients were moderately correlated with total IgE ($r = 0.341$, $P = 0.006$, Fig. 1) and had no correlation with eosinophil ($r = 0.135$, $P = 0.378$).

3.4. Association of cow milk-specific IgG and cow milk-specific IgE in CMA patients

Among the 40 CMA patients with lifted cow milk IgE antibody level, 28 (70.0%) present highly sensitive reactivity to cow milk IgG antibody, along with each six (15.0%) of moderate sensitive reactivity and mild sensitive reactivity to cow milk IgG antibody (Table 6).

Considering the 28 CMA patients with high sensitive milk-specific IgE reaction, their milk-specific IgG reactivity was highly sensitive too. Ten of them showed high cow milk-specific IgE concentration, 13 was moderate, and five was low (Table 6). Pearson correlation analysis was used to analyze the relationship between serum cow milk-specific IgE

and serum cow milk-specific IgG. Cow milk-specific IgE in CMA patients was moderately correlated with cow milk-specific IgG ($r = 0.415$, $P = 0.001$, Fig. 2).

4. Discussion

During the last 100 years, the advent of pasteurization and improvement of hygiene in farming lead to the dramatic increase in consumption of cow milk-based products by infants and breastfeeding mothers [15]. Despite the numerous health benefits of milk, there are potential detrimental effects, such as gastrointestinal complaints, colic, eczema, rhinitis, asthma, and anaphylaxis [16]. The major disorders, including late-onset CMA, are localized to the gastrointestinal tract. The most frequent symptoms among the common manifestations of CMA are gastrointestinal, which accounts for 50–75% of CMA patients [17]. In our study, all patients presented diarrhea, which is usually seen among the delayed symptom, but can also be immediate. Therefore, a reliable laboratory test on this disease is required.

In CMA patients with diarrhea symptom, degranulation of intestinal mast cells releases mediators acting on the gastrointestinal epithelium, which is an important mechanism for type I immunological reaction in CMA [18]. Determination of the total serum IgE concentration may be useful for checking the global allergic status of CMA patients [19]. In our study, out of the 65 CMA patients, 36 (55.4%) had total serum IgE antibodies elevated. The positive rate of total IgE in CMA patients was significantly higher than that in controls. It is unclear whether the milk-specific IgE antibodies contribute a considerable portion to the total IgE level in CMA patients; however, regardless of this, a moderate correlation was observed between the total and cow milk-specific IgE values in our observation. The serum milk-specific IgE was used to evaluate milk sensitization. In our study, 40 out of the 65 (61.5%) CMA patients had cow milk-specific IgE antibodies elevated. Most experimental results showed moderate cow milk-specific IgE reaction, followed by low and high reaction. This result indicated that the cow milk-specific IgE levels were presented in CMA patients with delayed symptom. Cow milk-specific IgE tests are very useful for detecting their presence, which is a sign of allergic sensitization. Fluorescence-labeled antibody assays have parallel sensitivity when compared with skin prick test, and the absolute levels of milk-specific IgE antibodies may directly correlate with the likelihood of

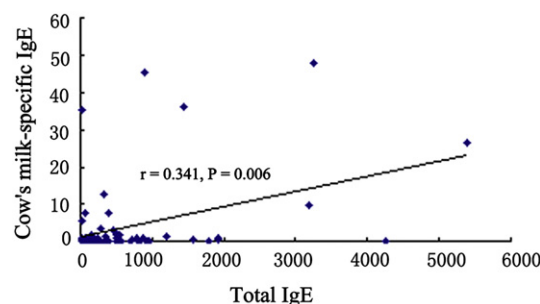


Fig. 1. Sera from 65 CMA patients were analyzed for the correlation between total IgE and cow milk-specific IgE. There was a significantly quantitative correlation between the levels of total IgE and cow milk-specific IgE ($r = 0.341$, $P = 0.006$).

Table 5
Cow milk-specific IgE in younger and adult patients.

Study group (year)	Cow milk-specific IgE-positive cases (n)	Cow milk-specific IgE concentration (U/mL)
≤ 18	32	5.842 ± 12.29
> 18	8	$0.641 \pm 1.603^*$
Total	40	4.161 ± 10.41

* The cow milk-specific IgE concentrations in younger patients (age ≤ 18 years old) were significantly higher than those in adult patients (age > 18 years old), ($t = 2.759$, $P = 0.008$).

Table 6

The changes of serum cow milk-specific IgE in CMA patients with different cow milk-specific IgG concentrations.

	CMA patients cases (n)	Spec. IgE negative cases (n)	Spec. IgE positive cases (n)				The positive rates of spec. IgE (%)
			Total	Low reaction	Moderate reaction	High reaction	
Milk-specific IgG 50–100 U/mL mild sensitive reactivity	15	9	6	3	3	0	15.0%
Milk-specific IgG 100–200 U/mL moderate sensitive reactivity	17	11	6	5	1	0	15.0%
Milk-specific IgG > 200 U/mL highly sensitive reactivity	33	5	28	5	13	10	70.0%
Total	65	25	40	13	17	10	100%

The positive rates of cow milk-specific IgE at three levels were significantly different among milk-specific IgG mild sensitive reactivity group, moderate sensitive reactivity group and highly sensitive reactivity group ($\chi^2 = 90.75$, $P = 0.000$).

Spec.: specific.

clinical reactivity when compared with oral food challenges for the identification of foods provoking IgE mediated food allergy [20]. In the present study we employed electrochemiluminescence immunoassay method, which was more sensitive than the fluorescence-labeled antibody assays [21].

The diagnostic value of specific IgG antibodies against cow milk proteins (CMP) is controversial because the production of these antibodies is a normal physiologic response following exposure to CMP [22]. Hochwallner et al. found that cow milk protein intolerance patients couldn't be distinguished from persons without cow milk protein intolerance on the basis of IgG subclass or IgA antibody levels to purified recombinant alpha S1-casein, alpha S2-casein, beta-casein, kappa-casein, alpha-lactalbumin, and beta-lactoglobulin [23]. Anthoni et al. found that milk protein IgG but not milk IgA seemed to be associated with self-reported milk-induced gastrointestinal symptoms [22]. In our study, serum cow milk-specific IgG was elevated in 65 CMA patients, while the total IgG levels were increased in 20 patients. These results suggested that the changes of cow milk-specific IgG couldn't always cause fluctuations of the total IgG concentration. Milk specific IgG in serum accounts for only a small part of total IgG in CMA patients. Therefore, CMA couldn't be diagnosed based on total IgG in clinical work. IgG is divided into 4 subclasses, IgG1, IgG2, IgG3 and IgG4. The diagnostic value of IgG subclass antibodies against different CMP is uncertain. Our data revealed that the IgG3 recorded the highest positive rate, followed by IgG2, IgG1, and IgG4 sequentially. These results were in accordance with previous studies reporting that milk casein elevated the IgG3 and IgG1 levels [23,24]. Hvatum pointed out that IgG3 and IgG1 antibodies were potentially inflammatory. They could enhance intestinal permeability and increase exposure to food antigens, thereby triggering the occurrence and development of type I allergies [25]. In addition, milk α -lactalbumin and β -lactoglobulin could elevate IgG4 levels [26]. IgG4 acts on mast cells and basophils, which are non-complement-fixing and functionally monovalent [27], and appears to be associated with IgE in a Th2-immunoregulatory fashion [28]. Therefore, IgG4 is involved in type I allergies [29]. Cow milk-specific IgG antibodies in CMA patients may be involved in type I allergic reaction.

In our study, we also found that patients with high cow milk-specific IgG concentration also had high cow milk-specific IgE concentration. Cow milk-specific IgE concentration in CMA patients was positively correlated with cow milk-specific IgG concentration ($r = 0.415$, $P = 0.001$). This correlation indicated that cow milk-specific IgE antibodies in CMA patients coexisted with cow milk-specific IgG antibodies, and IgE-mediated allergic immunity existed. Eosinophil number was further calculated. The findings indicated that absolute eosinophil number in 10 (15.4%) out of the 65 CMA patients was elevated. This result was consistent with previous studies reporting a similar trend for eosinophils and intraepithelial eosinophils and lymphocytes in the duodenal samples [30]. The present observation revealed a close correlation existing between the production of IgE and the levels of IgG antibodies against CMP, which may imply a close relationship between the synthesis of IgE and IgG, as previously suggested by Lilja et al. [31].

CMA patient with diarrhea symptom may be associated with a Th2-type of immune response, and linked to cellular IgG immune responses. Cow milk-specific IgE and IgG detection only play an auxiliary role in the diagnosis of CMA, which must be based on the clinical response to cow milk elimination and provocation, as recommended by Alessandro Fiocchi et al. [14].

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Ethical approval

The present study was approved by the Institutional Ethics Committee of Zhongshan Hospital, Medical College of Xiamen University, and was in compliance with the national legislation and Declaration of Helsinki guidelines. The written patient consents were obtained according to the institutional guidelines.

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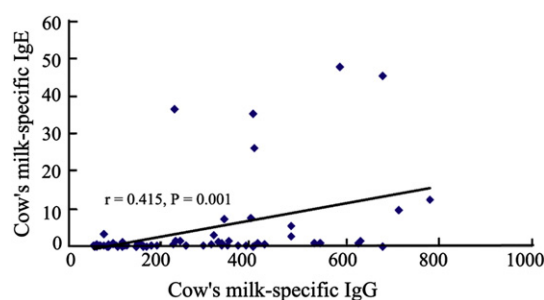


Fig. 2. Sera from 65 CMA patients were analyzed for the correlation between cow milk-specific IgG and cow milk-specific IgE. There was a significantly quantitative correlation between the levels of cow milk-specific IgG and cow milk-specific IgE ($r = 0.415$, $P = 0.001$).

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