

Usefulness of Lymphocyte Stimulation Test for the Diagnosis of Intestinal Cow's Milk Allergy in Infants

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Key Words

Cow's milk allergy • Infants • Cell-mediated hypersensitivity • Lymphocyte stimulation test • κ -Casein

Abstract

Background: Bottle-fed infants sometimes develop intestinal cow's milk allergy (ICMA). Because cow's milk-specific IgE antibody (CM-IgE) levels are normal, the lymphocyte stimulation test (LST) has been proposed as an alternative diagnostic test for ICMA. The present study evaluated the diagnostic value of LST in a large number of patients with ICMA in Japan. **Methods:** Ninety-six infants who developed intestinal symptoms after ingestion of cow's milk formula and showed remission of symptoms after elimination of this food were enrolled as patients with probable ICMA. Seventy-two subjects with normal CM-IgE levels and a positive result in an oral food challenge test (OFCT) for cow's milk formula were diagnosed with ICMA. Another 10 infants with normal CM-IgE levels and a negative OFCT result were diagnosed with nonspecific intestinal symptoms (NIS). The status of cell-mediated immunity against cow's milk proteins was estimated by LST for κ -casein. **Results:** In the 72 patients with ICMA (38 boys and 34 girls), the median age at onset was 9 days. Sixty-two of 72 (86.1%) patients with ICMA tested positive in the LST for κ -casein. In contrast, only 2 of the 10 NIS infants test-

ed positive. The incidence of a positive LST result was significantly higher in the ICMA group than in the NIS group ($p < 0.0001$). The area under the receiver-operating characteristic curve for this test was as high as 0.856. **Conclusions:** This study strongly suggests that the LST for κ -casein is a useful diagnostic test for ICMA.

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Introduction

Although cow's milk (CM) is a popular food, it is also a major food allergen. In developed countries, the prevalence of CM allergy is approximately 2–5% [1]. CM allergy is usually mediated by IgE antibodies for CM (CM-IgE), which trigger an immediate allergic reaction. However, CM formula also causes intestinal symptoms such as vomiting, bloody stool and diarrhea in a non-IgE-mediated manner in neonates and young infants [2–5], and these are here collectively referred to as intestinal CM allergy (ICMA). The incidence of ICMA was estimated to be 0.21% in Japan [6].

Because CM-IgE values are usually normal, serological tests or skin prick tests are of no or little value for the diagnosis of the disease. In contrast, the atopy patch test was shown to be a useful tool for the diagnosis of ICMA

Table 1. Profile of subjects

Item	ICMA	IgE(+)-ICMA	NIS
Subjects (male/female)	72 (38/34)	14 (7/7)	10 (9/1)
Age at onset	9 d (0 d to 6 m)	7 d (0 d to 1 m)	7 d (3 d to 6 m)
Symptoms, %			
Bloody stool	55.6	71.4	60.0
Vomiting	51.2	71.4	50.0
Diarrhea	48.6	42.9	30.0
Distension	6.9	0.0	20.0
CM-IgE (>0.34 UA/ml)	0.0	100 ^a	0.0
κ-casein-LST (>180)	86.1	78.6	20.0
Age at LST, months	1 (0–11)	1 (0–7)	4.5 (0–9)
Length of asymptomatic period before LST	11.5 d (3 d to 10 m)	13 d (8 d to 7 m)	18.5 d (7 d to 4 m)

The profile of ICMA, IgE(+)-ICMA, and NIS patients are shown. Age at onset, age at LST and the length of the asymptomatic period before LST are shown as the median and range (in parentheses). IgE(+)-ICMA = ICMA with a positive test result for CM-IgE; d = day; m = month; κ-casein-LST = lymphocyte stimulation test for κ-casein. ^a The median level of CM-IgE was 1.36 UA/ml (range 0.35–18.92).

[7]. In addition, a CM protein-specific lymphocyte stimulation test (LST), a common method to estimate cellular immunity, showed elevated values in patients with ICMA [8, 9]. The immunological mechanism of ICMA is therefore thought to be cell-mediated hypersensitivity to CM proteins [10]. However, the value of LST as a diagnostic test for ICMA is unclear [11].

In the present study, LST was evaluated as a diagnostic test for ICMA in a large number of patients in Japan.

Subjects and Methods

Subjects

Ninety-six infants who developed intestinal symptoms after ingestion of CM formula and showed remission of symptoms after elimination of this food were enrolled in the study from April 1, 2001, to December 31, 2009. The exclusion criteria were infectious, hematological, genetic, metabolic or surgical disorders, necrotizing enterocolitis, very low birth weight infants (<1,500 g) and untreated congenital malformations.

Among the 96 subjects enrolled in the study, 86 had a positive oral food challenge test (OFCT) result (fig. 1; table 1). Seventy-two subjects with normal CM-IgE were diagnosed with ICMA, whereas 14 subjects with positive CM-IgE were classified as IgE(+)-ICMA. Ten subjects with a negative OFCT result were diagnosed with nonspecific intestinal symptoms (NIS).

Ten subjects (6 boys and 4 girls) with gastrointestinal problems due to known etiologies such as galactose intolerance, infections and surgical disorders were included as negative controls. The median age at the LST examination was 2.5 months (range 0–9). None of the control subjects had a positive CM-IgE result, IgE-mediated food allergy or atopic dermatitis.

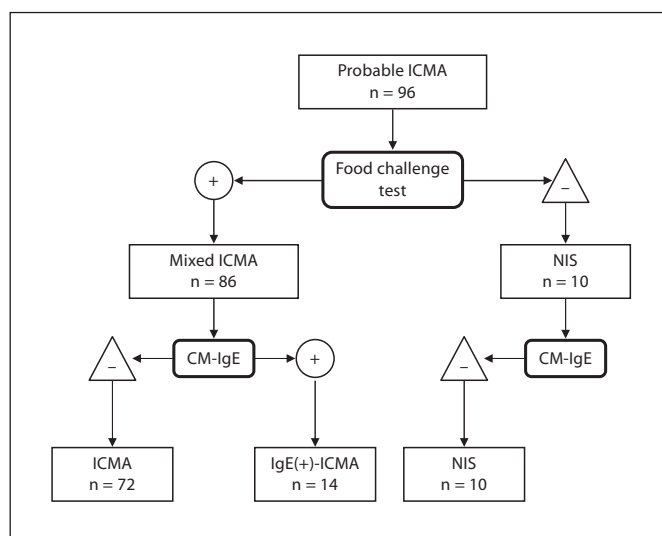


Fig. 1. Schematic representation of the diagnostic procedure. IgE(+)-ICMA = ICMA with a positive test result for CM-IgE.

Written informed consent was obtained from the parents before the study. The institutional approval for the study was obtained by the regional ethical committee.

Lymphocyte Stimulation Test

The LST was performed as described previously with some modifications [12]. In brief, peripheral blood mononuclear cells were resuspended at 1×10^6 cells/ml in RPMI1640 growth medium supplemented with 10% human AB serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Aliquots (0.5 ml) of the cell suspension were placed in a 48-well culture plate.

Table 2. Sensitivity of LST for various CM proteins

Study No.	Subjects	Antigens	Sensitivity
1	30	κ -casein crude extract of CM	90.0 60.0
2	42	κ -casein β -lactoglobulin	88.1 81.0
3	19	κ -casein α -lactalbumin	89.5 84.2
4	31	κ -casein lactoferrin	83.9 77.4
5	12	κ -casein α -casein β -casein	83.3 50.0 58.3

The sensitivity of the LST for κ -casein in diagnosing ICMA was compared with that for various purified CM proteins and a crude extract of CM.

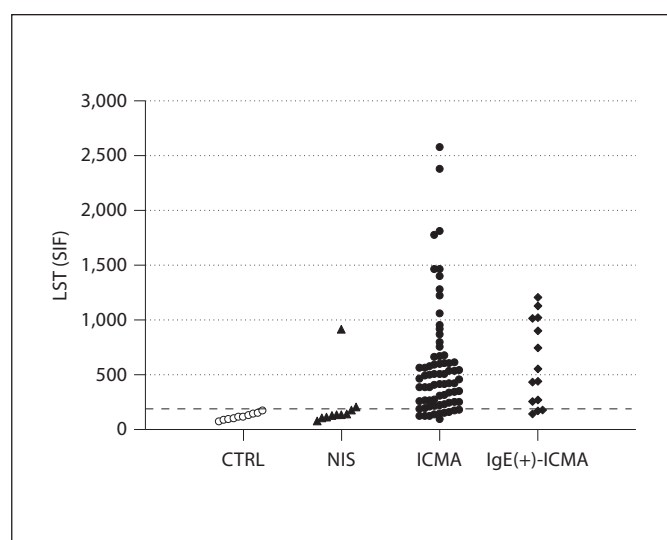


Fig. 2. LST for κ -casein in various subjects. The result of the LST for κ -casein in various subjects is shown. CTRL = Control subjects; IgE(+)-ICMA = ICMA with a positive test result for CM-IgE. The dotted line indicates the upper limit of the normal range.

Proliferation of peripheral blood mononuclear cells stimulated with κ -casein increases in a dose-dependent manner and reaches a maximum at a concentration of 100 $\mu\text{g/ml}$ [unpubl. observation]. After addition of 100 $\mu\text{g/ml}$ κ -casein (Sigma-Aldrich), cells were cultured for 6 days at 37°C in an atmosphere containing 5% CO_2 . Subsequently, the percentage of S-phase cells was quantified by flow cytometry (Epics Elite; Beckman Coulter). The LST

result was expressed as stimulation index measured by flow cytometry (SIF), where $\text{SIF} = (\text{percentage of S-phase cells in a stimulated sample} / \text{percentage of S-phase cells in an unstimulated sample}) \times 100$.

Endotoxins often contaminate preparations of CM proteins and cause nonspecific cell proliferation [13, 14]. In the present study, the concentration of endotoxins in the cell culture medium containing 100 $\mu\text{g/ml}$ κ -casein was less than 10 pg/ml.

The LST for κ -casein was considered to be positive when the SIF was higher than 180, which corresponds to the mean + 2 SD in control subjects (fig. 2). This test was not affected by either the age at onset of ICMA or the age at the time of examination in infants younger than 1 year (data not shown).

Comparison of the Sensitivity of the LST for Various CM Proteins

The sensitivity of the LST for κ -casein in the diagnosis of ICMA was compared with the LST for various CM proteins. The LST showed a higher sensitivity for κ -casein than for the crude extract of CM (table 2). The sensitivity of the LST for κ -casein in diagnosing ICMA was not inferior to either the LST for β -lactoglobulin, α -lactalbumin, lactoferrin, α -casein or β -casein (table 2).

OFCT and IgE Antibody Test

An OFCT was performed by feeding CM formula after intestinal symptoms had subsided (table 3). Most subjects were tested with the same volume of CM formula that they had been fed before the onset of the disease (regular procedure). To avoid severe reactions that require medical intervention such as intravenous fluid [5, 15, 16], OFCT was initiated in 17.8% (19/96) of subjects with a reduced volume of CM formula (5–20 ml), which was gradually increased if no symptoms developed (step-by-step procedure). If the intestinal symptoms were reproduced, then the test was considered to be positive.

CM-IgE was detected by the CAP-FEIA system.

Statistical Analysis

The statistical significance of the differences in the incidence of a positive LST result was determined by Fisher's exact test. The result was considered to be statistically significant if the p value was less than 0.05. Receiver-operating characteristic (ROC) analysis was performed using Excel Statistics 2008 for Microsoft Windows (SSRI).

Results

Clinical Features of ICMA

There was no apparent sex disparity in patients with ICMA (table 1). The median age at onset was 9 days (range 0 days to 6 months). Bloody stool, vomiting and diarrhea were observed in 55.6, 51.2 and 48.6% of patients, respectively. The incidence of intestinal symptoms in individual patients was as follows: bloody stool alone, 16.7% (12/72); vomiting alone, 16.7% (12/72); diarrhea alone, 15.3% (11/72); vomiting and diarrhea, 11.1% (8/72); bloody stool and vomiting, 16.7% (12/72); bloody stool and diar-

Table 3. Results of OFCT

Item	ICMA	IgE(+)-ICMA	NIS
Subjects	72	14	10
Age at OFCT	1 m (3 d to 12 m)	13.5 d (5 d to 6 m)	2 m (13 d to 9 m)
Asymptomatic period before OFCT	7 d (2 d to 10 m)	5 d (2 d to 6 m)	12 d (4 d to 4 m)
Time of reactions, %			
<1 h	4.2	7.2	
1–24 h	76.4	71.4	
>24 h	19.4	21.4	
Symptoms, %			
Vomiting	40.3	57.1	
Bloody stool	40.3	35.7	
Diarrhea	33.3	28.6	

The results of the OFCT in subjects with ICMA, IgE(+)-ICMA and NIS are shown. Age at OFCT and the length of the asymptomatic period before OFCT are presented as the median and the range (in parentheses). IgE(+)-ICMA = ICMA with a positive test result for CM-IgE; m = month; d = day.

rhea, 12.5% (9/72); bloody stool, vomiting and diarrhea, 9.7% (7/72) (table 4).

Oral Food Challenge Test

The median age of patients with ICMA at the time of the OFCT was 1 month (table 3). An OFCT was performed after 7 days of an asymptomatic period. A response developed in 80.6% (58/72) of IMCA patients within 24 h after the oral challenge. Among them, 3 patients (4.2%) developed symptoms within 1 h. Intravenous fluid transfusion was performed in the case of 19 (26.4%) patients because of severe intestinal symptoms, mainly vomiting. Intravenous fluid transfusion was performed in 23.1% (3/13) of patients in the step-by-step procedure group and in 27.1% (16/59) of patients in the regular procedure group.

LST for κ -Casein in Patients with ICMA

Sixty-two of 72 (86.1%) patients with ICMA had a positive LST result for κ -casein, whereas only 2 of 10 (20%) subjects in the NIS group had a positive result (fig. 2; table 1). The incidence of a positive LST result was significantly higher in patients with ICMA than in those with NIS ($p < 0.0001$). The area under the ROC curve was 0.856 for the LST for κ -casein in the differentiation of ICMA from NIS (fig. 3).

Classification of ICMA Patients by Individual Symptoms

The patients with ICMA were classified into 3 groups according to the type of individual intestinal symptoms

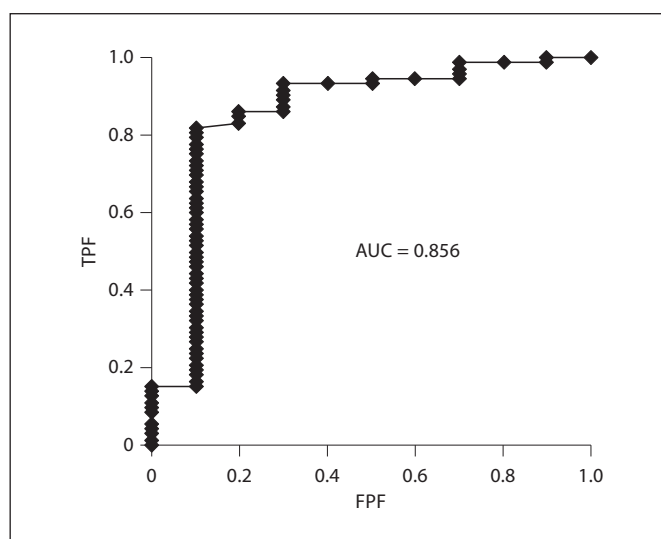


Fig. 3. ROC curve for LST for κ -casein. The ROC curve for the LST for κ -casein in the differentiation of ICMA from NIS is shown. The area under the ROC curve (AUC) was 0.856. TPF = True-positive fraction; FPF = false-positive fraction.

(table 4). Group I, which was consistent with proctitis, showed bloody stool alone and represented 16.7% (12/72) of the patients; group II was consistent with enterocolitis, which was characterized by vomiting and/or diarrhea, and accounted for 43.1% (31/72); group III, which was composed of the rest of the patients who showed bloody stool plus vomiting and/or diarrhea, accounted for 38.9% (28/72) of the patients.

Table 4. Classification of patients by individual symptoms

Group	Symptoms	Subjects	Age at onset		κ -casein-LST (>180)	
I proctitis	BS	12 (16.7%)	21 d (1 d to 3 m)	21 d (1 d to 3 m)	83.3%	83.3%
II enterocolitis	Vo	12 (16.7%)	7 d (1 d to 3 m)	26 d (1 d to 6 m)	91.7%	87.1%
	D	11 (15.3%)	1 m (8 d to 6 m)		72.7%	
	Vo+D	8 (11.1%)	19 d (4 d to 2 m)		100%	
III BS+ α	BS+Vo	12 (16.7%)	6 d (0 d to 1 m)	7 d (0 d to 4 m) ^a	91.7%	85.7%
	BS+D	9 (12.5%)	8 d (0 d to 4 m)		66.7%	
	BS+Vo+D	7 (9.7%)	7 d (0 d to 1 m)		100%	

Patients with ICMA were classified into 3 groups according to individual intestinal symptoms. The age at onset and the incidence of a positive LST result for κ -casein were compared among the 3 groups. A patient with abdominal distension alone was excluded. Age at onset is shown as the median and the range (in parentheses). BS = bloody stool; d = day; m = month; Vo = vomiting; D = diarrhea. ^a Significantly earlier than in group II.

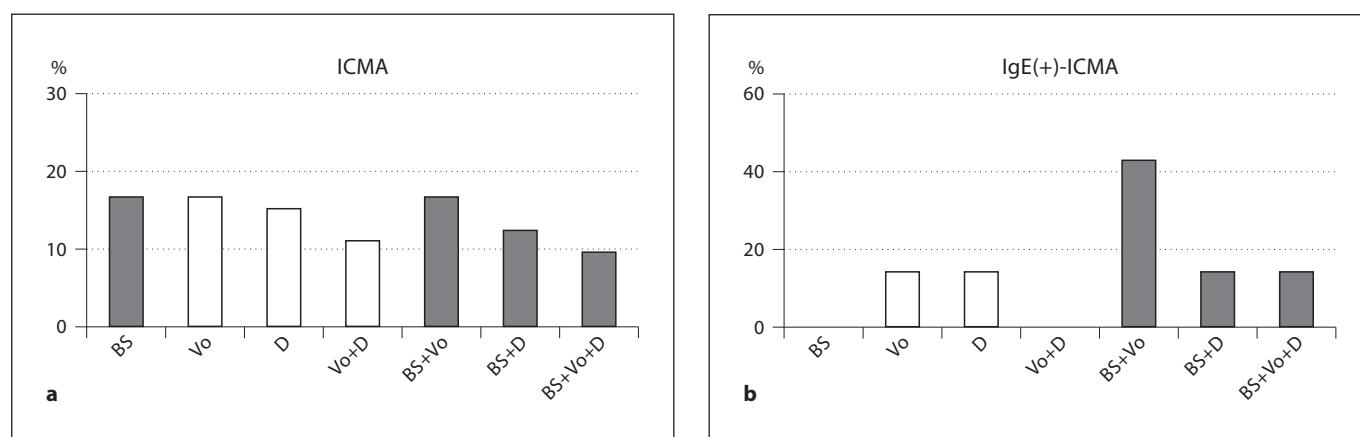


Fig. 4. Comparison of individual symptoms between ICMA and IgE(+)-ICMA. Intestinal symptoms were compared between patients with ICMA (a) and those with IgE(+)-ICMA (b). A patient with abdominal distension alone was excluded. IgE(+)-ICMA = ICMA with a positive test result for CM-IgE; BS = bloody stool; Vo = vomiting; D = diarrhea.

The age at onset was significantly earlier among group III than among group II patients ($p < 0.01$). The age at onset in group I was comparable to that in group II and later than in group III, although the difference was not significant. The incidence of positive κ -casein-LST was not significantly different among patients in groups I, II and III (table 4).

Features of IgE(+)-ICMA

In the present study, 14 subjects were defined as IgE(+)-ICMA (table 1). The median level of CM-IgE was 1.36 UA/ml (range 0.35–18.92). The age at onset and the percent-

age of positive kappa-casein-LST in patients with IgE(+)-ICMA did not differ from those in patients with ICMA. Patient symptoms showed a high incidence of the combination of bloody stool and vomiting (42.9%, 6/14), followed by diarrhea alone (14.3%, 2/14), vomiting alone (14.3%, 2/14), bloody stool and diarrhea (14.3%, 2/14), and bloody stool, vomiting and diarrhea (14.3%, 2/14) (fig. 4). Hives or anaphylaxis was not observed in the IgE(+)-ICMA patients despite a positive CM-IgE level. The OFCT response time did not differ between the ICMA and IgE(+)-ICMA patients (table 3).

Discussion

In the presence of normal CM-IgE levels, the diagnosis of ICMA must be based on symptoms, clinical course and OFCT results [2–5]. The lymphocyte stimulation test, which is commonly used to estimate cellular immunity, shows positive results when applied for CM proteins in patients with ICMA [8, 9]. However, the efficacy of the LST as a diagnostic tool for ICMA remains unclear [11]. The present study assessed the value of the LST as a diagnostic tool for ICMA in a large number of patients.

In addition, the results of the LST with various purified CM proteins and a crude extract of CM were compared to maximize the efficacy of this assay for the diagnosis of ICMA. κ -Casein was identified as the best stimulant because of the highest sensitivity for the diagnosis of ICMA. This result was consistent with those of a previous study concerning CM allergy complicated with atopic dermatitis [17].

A positive response in the LST for κ -casein was found in a significantly higher number of patients with ICMA than in those with NIS. Because the area under the ROC curve for the test was 0.856, the LST for κ -casein is estimated to be good for distinguishing ICMA from NIS. These results strongly suggest that the LST for κ -casein is an accurate diagnostic test for ICMA. The ability of the LST to detect an excess in the cellular immunity against CM proteins, which is indispensable for the diagnosis of cell-mediated food-induced allergic disorders, may encourage physicians to make an accurate diagnosis of ICMA.

Although ICMA is thought to be mediated by cellular immunity, the associated responses develop in most cases within 3 h after the food challenge [5, 15]. This response time is extremely short for a cell-mediated allergic reaction, which is known to require a time period longer than 24 h, and indistinguishable from IgE-mediated immediate allergic reactions. Similar results were obtained

with the OFCT in the present study, in which intestinal symptoms developed in most patients within 24 h after the ingestion of CM formula. The mechanisms that mediate such an early development of symptoms remain to be elucidated.

A recent classification of food allergies determined that non-IgE-mediated food-induced allergic disorders consist of proctitis and enterocolitis [10]. Proctitis is characterized by the presence of bloody stool alone, whereas enterocolitis includes vomiting and/or diarrhea. Indeed, in the present study, 16.7 and 43.1% of subjects appeared to show proctitis and enterocolitis, respectively (table 4). However, the symptoms of approximately one third of the patients were not consistent with either of these syndromes because they included bloody stool plus vomiting and/or diarrhea (table 4, group III). This group was also characterized by an earlier onset of the disease. It remains to be clarified whether this population of ICMA patients is peculiar to Japan or has been overlooked in studies conducted in Western countries.

Patients with positive CM-IgE who developed intestinal symptoms similar to those of ICMA but without hives or anaphylaxis, which is typical of IgE-mediated CM allergy, were included in the study. The incidence of patients showing a positive LST result for κ -casein, the age at onset and the OFCT response time were not different between patients with ICMA and those with IgE(+)-ICMA. However, there were obvious differences between these two groups in the individual symptoms, with a predominance of the combination of bloody stool and vomiting in patients with IgE(+)-ICMA but not in those with ICMA. These results suggest that IgE(+)-ICMA is not identical to either ICMA or IgE-mediated CM allergy. The pathogenesis and the prognosis of IgE(+)-ICMA should therefore be studied separately from ICMA, despite the fact that they share certain clinical and laboratory characteristics.

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