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รายงานวิจัยฉบับสมบูรณ์

การวัดระดับของ IgG และ IgG subclasses ต่อโปรตีนในนมวัว เพื่อใช้ในการวินิจฉัยภาวะแพ้นมวัว

รศ.พญ. พรรณทิพา ฉัตรชาตรี

30 มิถุนายน 2547

สัญญาเลขที่ TRG4580100

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ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์

จุฬาลงกรณ์มาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: TRG4580100

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บทนำ การแพ้นมวัวเป็นภาวะที่มีอุบัติการณ์เพิ่มขึ้นในประเทศไทย ส่วนหนึ่งเนื่องมาจากคนไทยมีการบริโภคอาหารเหมือนชาวตะวันตก ทำให้มีการบริโภคนมวัวเพิ่มขึ้น การดูแลรักษาผู้ที่แพ้นมวัวนั้น สิ่งสำคัญประการแรกคือ การวินิจฉัยที่ถูกต้อง ซึ่งปัจจุบันนี้การตรวจทางห้องปฏิบัติการเพื่อช่วยในการวินิจฉัยยังไม่ครอบคลุมการแพ้นมวัวชนิดต่างๆ การตรวจวัดระดับภูมิต้านทานชนิด IgG ต่อโปรตีนในนมวัว จะทำให้เข้าใจถึงการตอบสนองของร่างกายต่อโปรตีนในนมวัว และอาจใช้เป็นการตรวจเพื่อสนับสนุนการวินิจฉัยการแพ้นมวัวได้เพิ่มเติมจากวิธีการตรวจทางห้องปฏิบัติการที่มีอยู่

วิธีการ : ซีรั่มจากผู้ที่แพ้นมวัว 30 คน และกลุ่มควบคุม 30 คน ได้รับการตรวจหา IgG และ IgG subclasses ต่อโปรตีนในนมวัว โดยวิธี microprecipitin และ enzyme-linked immunosorbent assay (ELISA) ต่อโปรตีนชนิดต่างๆ ในนมวัว ได้แก่ casein, beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin.

ผลการศึกษา. ผลการตรวจด้วย microprecipitin reaction พบว่า casein and betalactoglobulin เป็นโปรตีนสำคัญที่ก่อให้เกิดการแพ้ในผู้ที่แพ้นมวัว นอกจากนี้ ยังพบว่า ผู้ที่แพ้นมวัว มีโอกาสให้ผลบวกต่อ whole cow's milk มากกว่ากลุ่มควบคุม. ซีรั่มของผู้ที่แพ้นมวัวส่วนใหญ่ (84%) ให้ผลบวกต่อโปรตีนในนมวัวอย่างน้อยหนึ่งชนิด ในขณะที่ซีรั่มของกลุ่มควบคุมส่วนใหญ่ (83%) ให้ผลลบต่อโปรตีนในนมวัวทุกชนิด และเมื่อแยกตามจำนวนของชนิดโปรตีนนมวัวที่ซีรั่มทำปฏิกริยาด้วย พบว่าซีรั่มของผู้ที่แพ้นมวัวทำปฏิกริยากับโปรตีนหลากหลายชนิดกว่ากลุ่มควบคุม ผลการตรวจ cow's milk protein specific and fraction specific IgG subclasses พบว่า ผู้ที่แพ้นมวัว มีระดับ IgG1 และ IgG4 ต่อโปรตีนในนมวัวสูงกว่ากลุ่มควบคุม ส่วน IgG2 และ IgG3 ต่อโปรตีนในนมวัวมีระดับต่ำในทั้งสองกลุ่ม และไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติ

บทสรุป. ระดับของ IgG และ IgG subclasses ต่อโปรตีนชนิดต่างๆ ในนมวัวมีความแตกต่างกันระหว่างผู้ที่แพ้นมวัวและคนปกติ การตรวจพบ IgG ต่อโปรตีนนมวัวในระดับที่สูง และการพบผลบวกของปฏิกริยา precipitin ต่อโปรตีนหลายๆ ชนิดในนมวัวสามารถใช้ประโยชน์ในการสนับสนุนการวินิจฉัยการแพ้นมวัว

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต ผลการวิจัยบ่งชี้แนวโน้มที่จะใช้การตรวจนี้ในการช่วยวินิจฉัยการแพ้นมวัวซึ่งเป็นโรคที่ผู้ป่วยมีอาการแสดงที่หลากหลาย การศึกษาโดยติดตามต่อเนื่อง ถึงระดับของ IgG ที่

เปลี่ยนแปลงไปในแต่ละช่วงของการดำเนินโรค เพื่อศึกษาถึงความเป็นไปได้ที่จะใช้การตรวจนี้ในการพยากรณ์โรคว่ามีแนวโน้มจะหายจากโรคนี้หรือไม่ จะทำให้การตรวจมีประโยชน์ในการดูแลผู้ป่วยเพิ่มขึ้น

Keywords: Cow's milk allergy, Cow's milk protein, Food allergy, Diagnosis

Abstract

Project Code : TRG4580100

Project Title : Cow's milk specific IgG and IgG subclasses measurement : Role for diagnosis of cow's milk allergy

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Project Period : July1, 2002-June 30, 2004

Background. Cow's milk allergy (CMA) has been increasingly common in Thailand with westernized dietary style and the increase in milk consumption of our population. Physicians taking care of the patients are faced with diagnostic difficulty since the diagnostic test currently available is not specific. Since food allergen-specific IgG may play an important role in pathogenesis of food allergic reactions, study of cow's milk protein-specific (CMP) and cow's milk protein fraction-specific (CMPF) IgG and IgG subclasses will lead to better understanding of this disorder. The information may be used to develop a more effective tool for the diagnosis of CMA. This will lead to early intervention and proper management and better quality of life for patients who suffer from this disorder.

Methods. Sera were collected form 30 patients with CMA and 30 controls. Microprecipitin technique was used to identify cow's milk protein fraction specific IgG. Enzyme-linked immunosorbent assay (ELISA) was used to measure IgG subclasses antibodies to major cow's milk allergens.

Results. Microprecipitin reaction revealed casein and betalactoglobulin to be the major antigenic proteins for patients with CMA. Patients with CMA had higher frequency of reaction to whole cow's milk than normal controls. The majority of patients (84%) had positive reaction to at least one fraction of cow's milk protein while most of the controls sera (83%) had negative precipitin reaction with all CMP fractions. When categorized the subjects according to the number of CMP fractions they reacted, sera from patients with CMA gave positive reaction to more fractions of cow's milk protein compared to control group. Analysis of cow's milk protein specific and fraction specific IgG subclasses showed that patients with CMA had significantly higher levels of IgG1 and IgG4 to CMP fractions when compared to controls. Low levels of IgG2 and IgG3 were observed in both groups and the difference was not statistically significant.

Conclusion. Cow's milk-specific IgG and IgG subclasses had distinct patterns between patients with CMA and normal subjects. The high levels of cow's milk specific antibodies and positive precipitin test to multiple protein fractions may have a diagnostic value in identifying patients with CMA.

Suggestion: This study suggested the clinical application of the tests for cow's milk-specific IgG and IgG subclasses in diagnosis of CMA. Follow up of the titers during the course of illness may have roles in predicting the prognosis and the probability to outgrow CMA.

Keywords: Cow's milk allergy, Cow's milk protein, Food allergy, Diagnosis

Introduction

The prevalence of atopic diseases is increasing worldwide with food allergy as the earliest manifestations. Up to 8% of children less than 3 years of age and approximately 2% of the adult population experience food-induced allergic disorders[1]. Prospective studies from several countries indicate that about 2.5% of newborn infants experience hypersensitivity reactions to cow's milk in the first year of life[2-4]. Survey questionaire revealed the prevalence of food allergy in 6-12 years old school children in Bangkok to be 3.89%[5]. Cow's milk allergy (CMA) is one of the most common causes of food allergy in children. It is expected to be increasingly common in Thailand with increase consumption of cow's milk in the diet. CMA is an important cause of protracted diarrhea in infancy. Cows' milk protein-sensitive enteropathy was found to be an important contributing cause of secondary sugar intolerance in young infants with acute infective enteritis[6, 7].

CMA has diverse manifestations which make it difficult to recognize and is sometimes overlooked. Because of the complex pathophysiologic mechanisms, reactions are classified into three groups; IgE-mediated, partially IgE-mediated, and non-IgE- mediated reactions. Heterogeneous immune pathophysiologic causes that underlie food allergic reactions remain to be elucidated. Cow's milk-induced allergic reactions can involve multiple organ systems[8]. Major target organs involved are the skin, the upper and lower respiratory tracts and the gastrointestinal tract. Manifestations may vary greatly. Symptoms range from itchy mouth, skin rash, diarrhea, poor weight gain to the life-threatening fatal food-induced anaphylaxis and death.

Once suspected the diagnosis of food allergy rested on laboratory testing, responses to food challenges and food exclusion. The diagnosis of FA can be extremely difficult, especially in cases with delayed reactions because the temporal relationship of food ingestion and the symptoms are not

obviously noticeable. Proper diagnoses require screening tests for evidence of cow's milk-specific antibodies and proof of reactivity through elimination diets and oral food challenges. Currently, the sensitive and specific laboratory tests to help in the diagnosis are lacking. Patients with suspected delayed reactions to cow's milk have to go through prolonged cow's milk elimination which can take as long as 12 weeks before diagnosis can be made. Improper diagnosis which includes both over-diagnosis and under-diagnosis can lead to growth failure and irreversible organ damage in patients with this disorder.

Cow's milk protein consists of four major allergens: casein, beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin. Even though cow's milk proteins have been well characterized chemically and physically, there are few data available, especially in humans, regarding their antigenic and allergenic properties. Caseins are the major allergens responsible for CMA. They account for about 80% of the total protein content in cow's milk. The role of cow's milk protein –specific (CMP), and cow's milk protein fraction-specific (CMPF) IgG in the diagnosis of CMA have not been well elucidated even though there have been some postulation about its role in delayed reactions. Determination of CMP IgG has been found to be of diagnostic significance in some studies [9-13], whereas it has not been confirmed by others [14, 15]. Most of the previous studies employed only one method of IgG measurement, and did not measure specific IgG to all major cow's milk allergens and its subclasses. Atopic dermatitis, one important atopic disorder usually associated with food allergy, associates with high levels of IgG subclass antibodies to beta-lactoglobulin in early childhood[16]. In children, it was found that the IgG response to bovine casein was largely restricted to IgG1 in contrast to the predominant IgG4 response to these antigens that is found in adults[17]. There have been evidences that antibodies that recognize different fractions of cow's milk and also antibodies that recognize different types of epitopes on the

same protein (conformation vs linear) may play a role in clinical course of CMA[18-23]. To elucidate the role of CMP-IgG and their diagnostic value, the antibodies need to be further studied in details with regard to their subclasses, fraction-specificity, and ability to form immune precipitate with the allergens.

Precipitin reaction is the test using the ability of an antibody to form immune precipitate to measure its concentration. The reaction has been use for the diagnosis of immune complex diseases such as hypersensitivity pneumonitis and allergic bronchopulmonary aspergillosis. It was found that patients' sera possessed non-precipitating serum factors most likely belong to the IgG3 immunoglobulin subclass, whereas the precipitating antibodies belong to the subclasses IgG1 and IgG2. This suggested that precipitin reaction mainly measure the activity of certain IgG subclasses more than others[24]. The role of IgG in pathogenesis of CMA may be better understood if the antibody that form immune complex in this reaction can be identified. Since this technique is simple to perform, it is a good candidate to develop as a screening test for CMA. However, the limitation of this method is its low sensitivity. In this study, we proposed to experiment with different reaction conditions to improve sensitivity of the test.

The objectives of this study include the identification of clinical significance of IgG antibody and IgG suclasses antibody to whole cow's milk protein ,and to protein fraction of cow's milk in CMA. We also utilize different laboratory methods to characterize CMP and CMPF IgG and IgG subclasses to evaluate their applications and potential to develop as a diagnostic tool for CMA.

Materials and methods.

Clinical assessment and specimen collection

All patients with suspected cow's milk allergy visiting the allergy clinic at the King Chulalongkorn Memorial Hospital were clinically assessed in details including history taking, physical examination and undergo food elimination and challenge to obtain definite clinical diagnosis. After the objectives of the study were explained to the patients and the consent was given, three to five ml. of peripheral blood was collected. Serum was separated and stored at –70 degree Celcius until used

CMP- and CMPF-specific IgG measurement

CMP- and CMPF-specific IgG and IgG subclasses to all four major cow's milk allergens (casein, beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin) were measured using enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA) for detection of IgG and IgG subclasses antibodies to major milk allergens

The determination of IgG subclass antibodies to major milk allergens including caseins (CS), beta-lactoglobulin (BLG), alpha-lactalbumin (ALA) and bovine serum albumin (BSA) were done. Microtitre plates were coated with each one of milk allergens. Free binding sites were blocked with 1% gelatin . Human serum samples or buffer without added serum (blanks) were added to duplicate wells; then monoclonal antibodies to the human IgG and IgG subclasses (IgG1, IgG2, IgG3, IgG4). Alkaline-phosphatase–conjugated rabbit anti-mouse IgG was added, and para-nitrophenylphosphate was used as substrate. The OD was read at 405 nm. Values were expressed as optical density ratio which is sample OD/calibrator OD.

Microprecipitation technique to detect CMP and CMPF- IgG fractions.

The microslide technique of double diffusion was employed. Agarose gel was casted on the microscopic slide. Patients' sera and extracts from four major cow's milk allergens were placed on the slides. After incubation, the precipitin lines were observed and recorded. Different reaction conditions will be utilized and compared in order to established the most suitable and sensitive methods of CMP-IgG detection

Statistical analysis

Data between groups were analyzed with Mann-Whitney U-test. Calculations were performed with SPSS for window. (SPSS Inc, Chicago, IL). P- values <0.05 were considered significant.

Results

There were 30 CMA patients enrolled. The age and clinical manifestations was shown in table 1. The most common manifestation of CMA in the studied group was in the gastrointestinal system. The age and gender of patients and control group was not statistically different.

Table 1 Clinical data of cow's milk allergic patients and controls

Number of children with CMA	30
Mean age (month)	5.5 (1-40)
Gender, F/M	10/20
Presenting symptoms %	
Gastrointestinal	55
Skin	20
Respiratory	10
More than one system	15
Controls	30
Mean age (month)	6.5 (1-48)
Gender, F/M	12/18

Microprecipitin reaction revealed casein (CS) and betalactoglobulin (BLG) to be the major antigenic proteins for patients with CMA. Among patients with CMA, 60% had positive precipitin reaction to CS, 50% to BLG, 33% to ALA and 27% to BSA. In normal control 3% had positive reaction to CS, 6% to BLG, 6% to BSA and none had positive reaction to ALA. Patients with CMA had higher frequency of reaction to whole cow's milk than normal controls and challenge negative patients. Eighty three percents of patients with CMA had positive reaction to whole cow's milk and 16% were negative to all protein fractions (figure 1). The majority of patients had positive reaction to more than one fractions of CMP with 40% reacted to two fractions and 26% reacted to three fractions of CMP (figure 2). Most of the controls sera (83%) had negative precipitin reaction with all CMP fractions. When categorized the subjects according to the number of CMP fractions they reacted, sera from patients with CMA gave positive reaction to more fractions of cow's milk protein compared to control group. The sera that reacted to two or three CMP fractions were from patients with CMA. None of the controls reacted to more than one CMP fraction (figure 3).

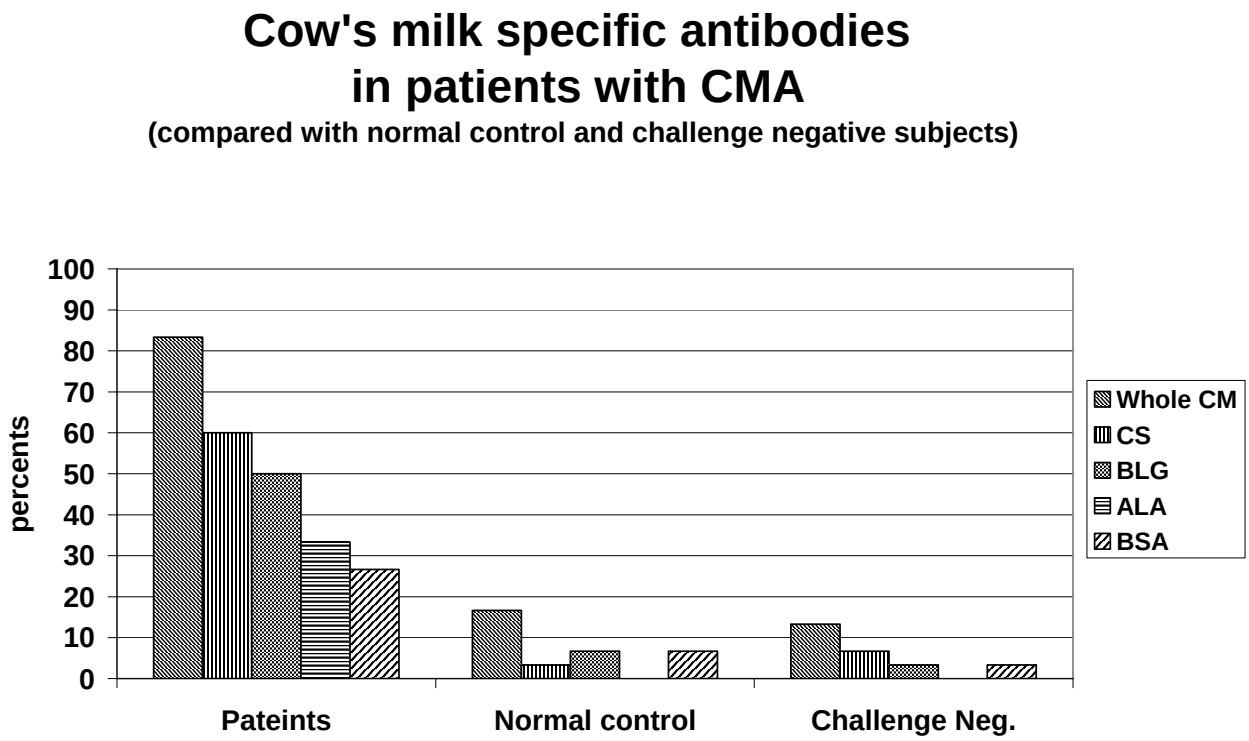


Fig 1. Cow's milk protein specific antibodies by precipitin test in patients with CMA compared to controls and challenge negative subjects

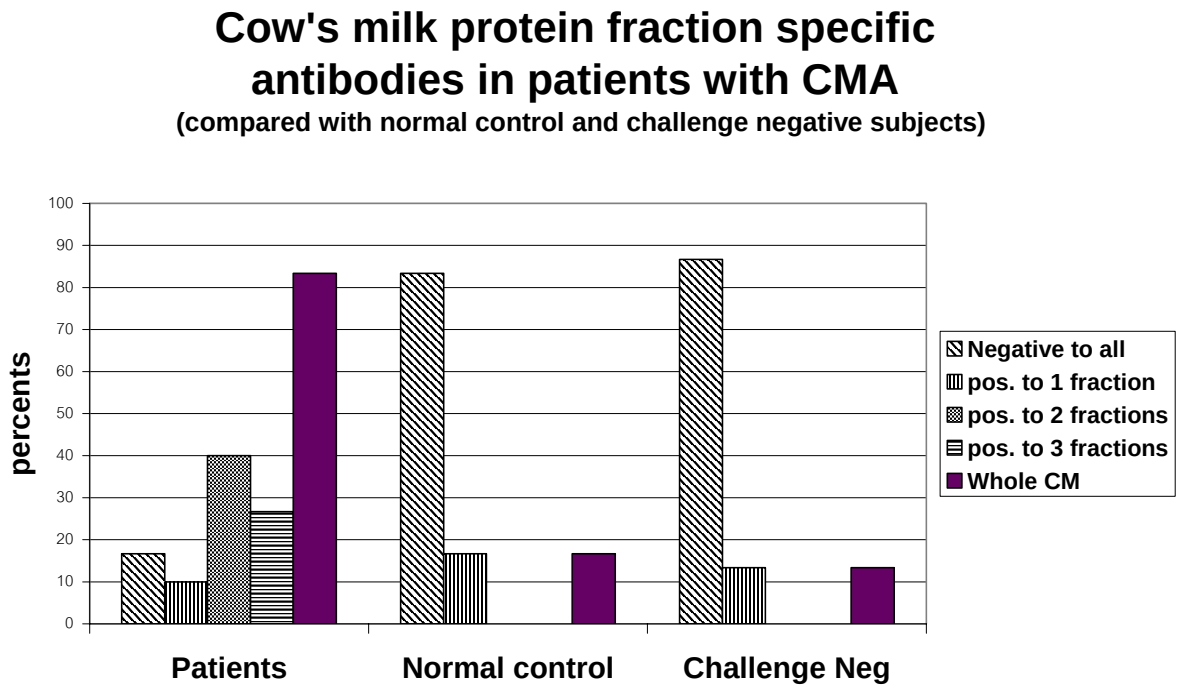


Fig 2 Cow's milk protein fraction specific antibodies by precipitin test in patients with CMA compared to controls and challenge negative subjects

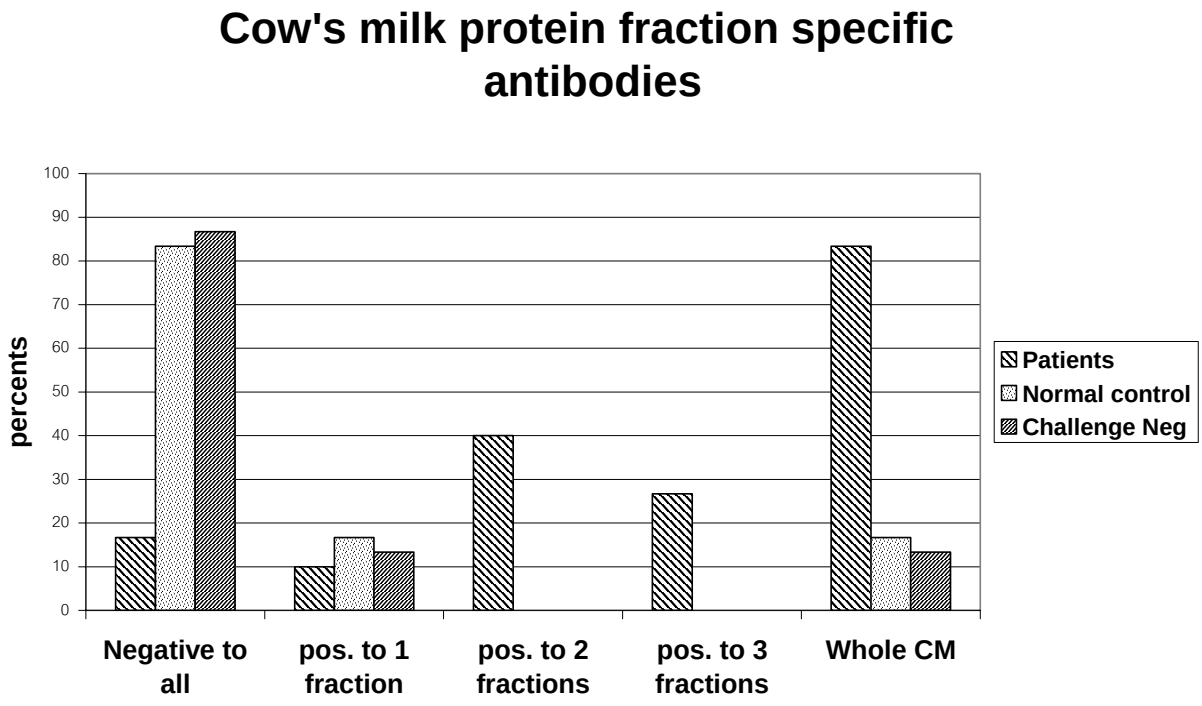


Fig 3 Cow's milk protein fraction specific antibodies by precipitin test

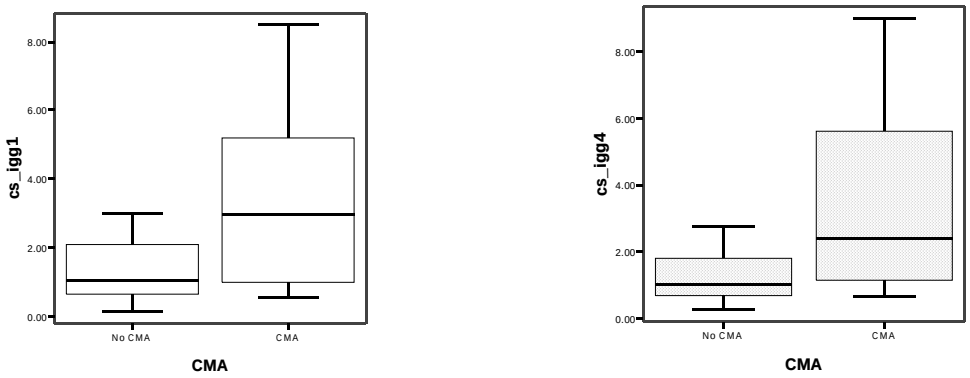
The groups were categorized by number of cow's milk protein fractions with positive reaction

Analysis of cow's milk protein specific and fraction specific IgG subclasses by ELISA, showed significant differences of IgG1 and IgG4 between patients with CMA and controls for all fraction of CMP ($p<0.05$) as shown in figure 4. Patients with CMA had higher levels of IgG1 and IgG4 to CMP fractions. IgG1 correlated with positive reaction in precipitin test while IgG4 did not correlated with the present of positive precipitin reaction. Low levels of IgG2 and IgG3 were observed in both groups and the difference was not statistically significant.

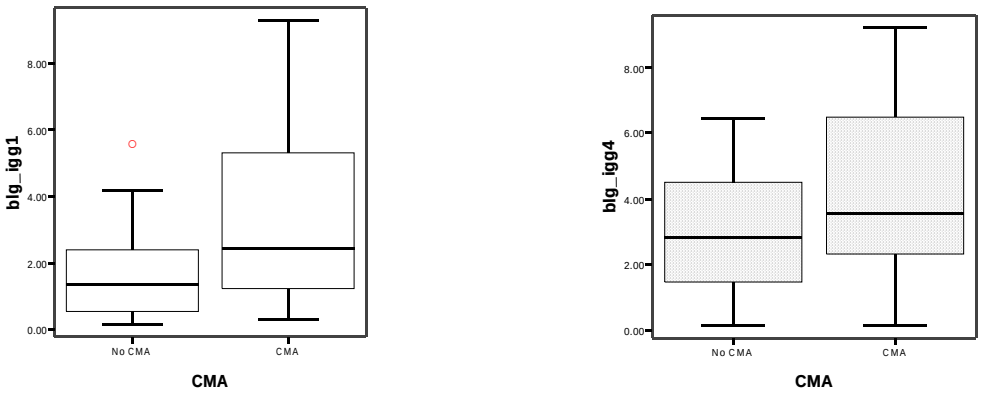
Fig. 4. Levels of IgG subclasses to different cow's milk protein fractions measured by ELISA in patients with CMA compared to control group.

Boxes represent the 25th , 50th and 75% percentiles and whiskers the 99th percentiles.

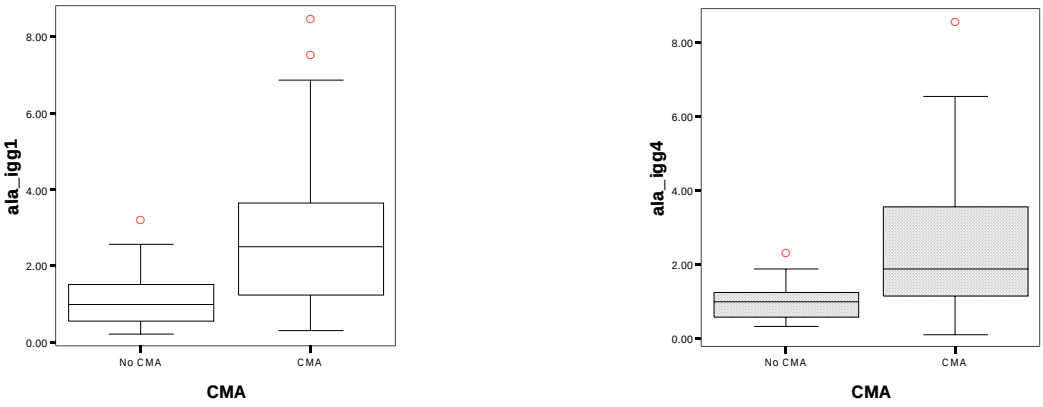
* *P* <0.05 when the levels of IgG1 or IgG4 of patients with CMA were compared to those of controls



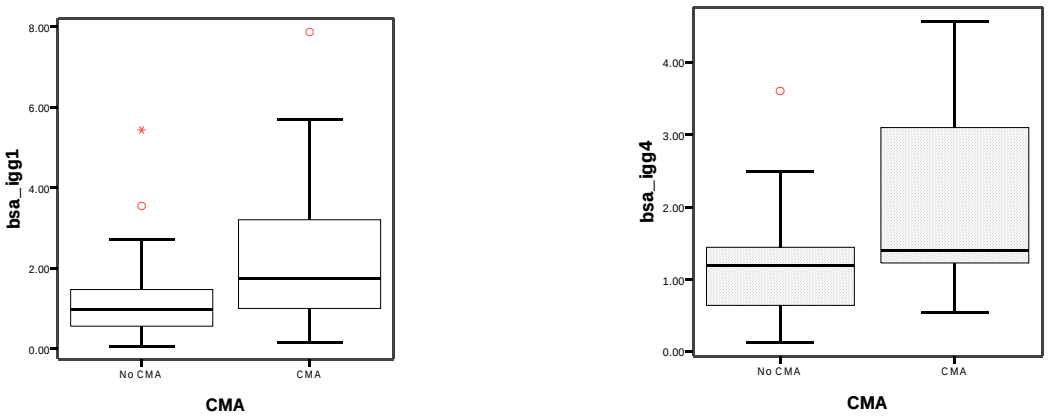
4.1 Levels of IgG subclasses (IgG1 on the left, and IgG4 on the right) to casein in normal control and CMA patients



4.2 Levels of IgG subclasses (IgG1 on the left, and IgG4 on the right) to beta-lactoglobulin in normal control and CMA patients



4.3 Levels of IgG subclasses (IgG1 on the left, and IgG4 on the right) to alpha-lactalbumin in normal control and CMA patients



4.4 Levels of IgG subclasses (IgG1 on the left, and IgG4 on the right) to bovine serum albumin in normal control and CMA patients

Discussion

The symptoms of CMA may mimic several other diseases. We sought to identify the significance of cow's milk protein specific IgG in patients with CMA. The diagnostic value of IgG antibodies to CMP is controversial because the production of those antibodies can be a normal physiologic response following exposure to cow's milk.

In the present study, we found casein and betalactoglobulin to be the predominant antigenic proteins for patients with CMA. The majority of patients with CMA gave positive precipitin reaction to CS or BLG while less than 10% of controls did. Positive precipitin reaction to multiple fraction of CMP was found more frequent in patients with CMA than in control group.

Measurement of cow's milk protein specific IgG subclasses revealed IgG1 and IgG4 to be the main subclasses that can be detected in sera of patients with CMA and in controls. The levels of cow's milk protein fraction specific IgG1 and IgG4 were significantly higher in patients with CMA compared to those of controls. Even though the present of IgG antibodies to cow's milk is the normal physiologic response to the ingestion of cow's milk, our patients with CMA produced significantly more milk specific antibodies than the controls. The increase in milk –specific antibodies may be a result of increased gut permeability lead to a greater passage of antigens into the circulation and the generation of a more intense antibodies response.

In summary, our study showed that cow's milk-specific IgG and IgG subclasses had distinct patterns between patients with CMA and normal subjects. The high levels of cow's milk specific antibodies and positive precipitin test to multiple cow's milk protein fractions may have a diagnostic value in identifying patients with CMA.

Acknowledgements

This work was supported by the Asahi Glass Foundation Fund and the grant from the Thailand Research Fund, Thailand.

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