

Cow's milk protein allergy: immunological response in children with cow's milk protein tolerance

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Summary

Objective: To study changes in immunological responses in patients with CMPA during symptomatic and asymptomatic episodes of cow's milk protein tolerance status.

Methods: 27 CMPA patients were enrolled and underwent diagnostic evaluation, including CM challenge test, skin prick test and specific IgE to CM. Blood samples were collected in two periods from those who became tolerant ($n = 13$) and those with persistent CMA ($n = 14$), in order to measure *in vitro* PBMC responses to cow's milk protein (IL-10, IFN- γ , IL-5), IgG4 to β -lactoglobulin, casein, BLG-IgG4/IgE ratio and the CAS-IgG4/IgE ratio.

Results: Seventy percent of CMPA patients in our study were male with a mean age at diagnosis of 8 months and mean age of onset of 3 months. The reaction time to CM ranged from within 7 minutes to within 14 days. Positive IgE-sensitization was defined as either a specific IgE to CM of more than 0.35 kUA/L ($N=11$) or SPTs positive for CM and/or fresh cow milk ($N=20$). Forty-eight percent of the patients ($n = 13$) could tolerate CM by 13.38 months (8 – 19 months). Mean specific-IgE levels to CM were 4.1 kUA/L (range 0.35 – 14.3 kUA/L). Determination of the cytokine (IL-10, IFN- γ , IL-5) response to BLG revealed significantly higher IL-10 levels during the tolerance phase (212.93 vs 142.46 pg/ml, $P = .011$). There was a significant increase in BLG-IgG4 and the BLG-IgG4/IgE ratio in the tolerance phase when compared to the symptomatic phase.

Conclusions: IL-10, BLG-IgG4 and the BLG-IgG4/IgE ratio were higher in CMPA patients during the tolerance phase compared to the symptomatic phase. (*Asian Pac J Allergy Immunol* 2013;32:171-7)

Key words: BLG-specific IgG4/IgE-CM ratio, CAS-specific IgG4/IgE-CM ratio, cow's milk protein allergy, cow's milk protein tolerance, Immunoglobulin G4

Abbreviations

CMP, CM	=	cow's milk protein, cow's milk
CMA, CMPA	=	cow's milk allergy, cow's milk protein allergy
DBPCFC	=	Double blind placebo control food challenge test
IgE	=	Immunoglobulin E
IL	=	Interleukin
Tregs	=	regulatory T cells
TGF	=	transforming growth factor
IgG4	=	Immunoglobulin G4
BLG	=	β -lactoglobulin
CAS	=	casein
PBMC	=	peripheral blood mononuclear cells
FTT	=	failure to thrive
UGIB	=	upper gastrointestinal bleeding
LGIB	=	lower gastrointestinal bleeding
SPT	=	skin prick test

Introduction

Cow's milk protein allergy (CMPA) is a common finding in pediatric practice and a comorbidity in atopic dermatitis patients.¹ The prevalence of the disease is approximately 2 to 3% of the general population.² The mechanism related to cow's milk protein allergy is classified into IgE mediated, non-IgE mediated or both types occurring simultaneously. Children can present with skin lesions, GI symptoms and respiratory symptoms.³

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Positive skin prick tests and/or serum specific IgE for CM can only be demonstrated in IgE mediated CMPA patients. Occasionally, atopic patch testing may be useful in diagnosing non IgE-mediated CMPA.⁴ The treatment of CMPA is avoidance of cow's milk and dairy products.⁵ Usually, the prognosis of CMPA is good. CMPA symptoms diminish when the patients grow up, mostly by 3 years old.³

CMPA is mediated by the stimulation of Th2 cells, with high production of interleukin (IL)-4, IL-5 and IL-13.⁶ Several studies have suggested that regulatory T cells (Tregs) play an important role in the suppression of Th2 function,⁷⁻⁸ thus, inhibiting the allergic reaction. IL-10 is a major regulatory cytokine of inflammatory responses which plays a key role in the induction and maintenance of anergic states.⁹ Moreover, the T-cell response to allergens entering by the mucosal route is actively suppressed by IL-10 in healthy subjects.¹⁰ The high IL-10 level obtained in the DBPCFC-negative group who had undergone previous oral challenge with positive results suggests a role for this cytokine in outgrowing food allergy, rendering this interleukin a useful tool in the diagnosis of food tolerance in previously food-allergic patients.¹¹

Furthermore, Immunoglobulin G (IgG) antibodies to food allergens are produced in both atopic and non-atopic children. Allergic symptoms and atopic sensitization are associated with high levels of specific IgG subclass antibodies to allergens, particularly IgG4. The production of IgE and IgG4 antibodies is regulated by similar mechanisms, e.g. IL-4 from Th2 cells induces both IgE and IgG4 switching in B-cells.¹² In contrast, IL-10 inhibits IgE production but up-regulates the secretion of IgG4, suggesting different ways to control the IgE and IgG4 production.¹³ Therefore, Tregs stimulate B cell to increase IgG4 and to suppress IgE. A previous study showed that the natural development of tolerance in patients with egg allergy was associated with an increase in ovalbumin-specific IgG4 level and a decrease in ovalbumin-specific IgE level.¹⁴ Ruiter et al.¹⁵ found that the maintenance of tolerance to cow's milk in atopic children and adults without CMPA was associated with elevated levels of specific IgG4. Additionally, Tomicic et al.¹⁶ reported the results of a study to identify immunological differences between infants with clinical signs of eczema and sensitization to food allergens before and after a 6-wk treatment period and at 4 years of age. Children sensitized to egg

and/or milk that could eat and drink the offending foods at 4 years of age had higher levels of IgG4 antibodies to ovalbumin and β -lactoglobulin and also higher IgG4/IgE ratios.

The aim of this study was to identify the changes in cytokine responses in patients with CMPA during symptomatic and asymptomatic periods.

Methods

Study Population

Subjects were recruited from the pediatric outpatient department at Ramathibodi hospital during the period from January 2010 to March 2011. The inclusion criteria were CMPA in patients between 0 – 5 years. 37 patients were suspected to have cow milk allergy (CMA). All the children underwent diagnostic evaluation, including cow's milk challenge test and skin prick tests or determination of specific IgE to CM. Blood samples were then collected in two periods, during episodes of symptoms and 6 month after avoiding cow's milk, in order to measure *in vitro* peripheral blood mononuclear cell (PBMC) cytokines responses (IL-10, IFN- γ , IL-5) to cow's milk protein, IgG4 to β -lactoglobulin (BLG), casein (CAS) and the IgG4/IgE ratio. The exclusion criteria were patients who had received intravenous or oral corticosteroids within the last 4 weeks before enrollment, patients with food allergy other than cow's milk and other co-morbid illnesses. Six patients with a negative cow's milk challenge test were excluded. The diagnosis of CMA was confirmed in 31 children, based on an open challenge test with cow's milk, skin prick tests or determination of specific IgE to cow's milk. After the elimination of cow's milk and dairy products from the diet for at least 6 months or at the age of one year, we repeated the diagnostic evaluation based on an open challenge test with cow's milk and laboratory investigations. There were four patients who deflated from follow-up at this time.

The 27 children with CMA were divided in two groups. Thirteen patients had recovered from CMA by the age of 13.4 months and 14 patients still had positive cow's milk challenge test results as demonstrated in Figure 1.

Skin prick test (SPT)

Skin prick tests were performed with cow's milk extract (ALK Abelló, INC.). Skin prick tests were considered positive if the wheal reaction was at least 3 mm. in diameter larger than that for a normal

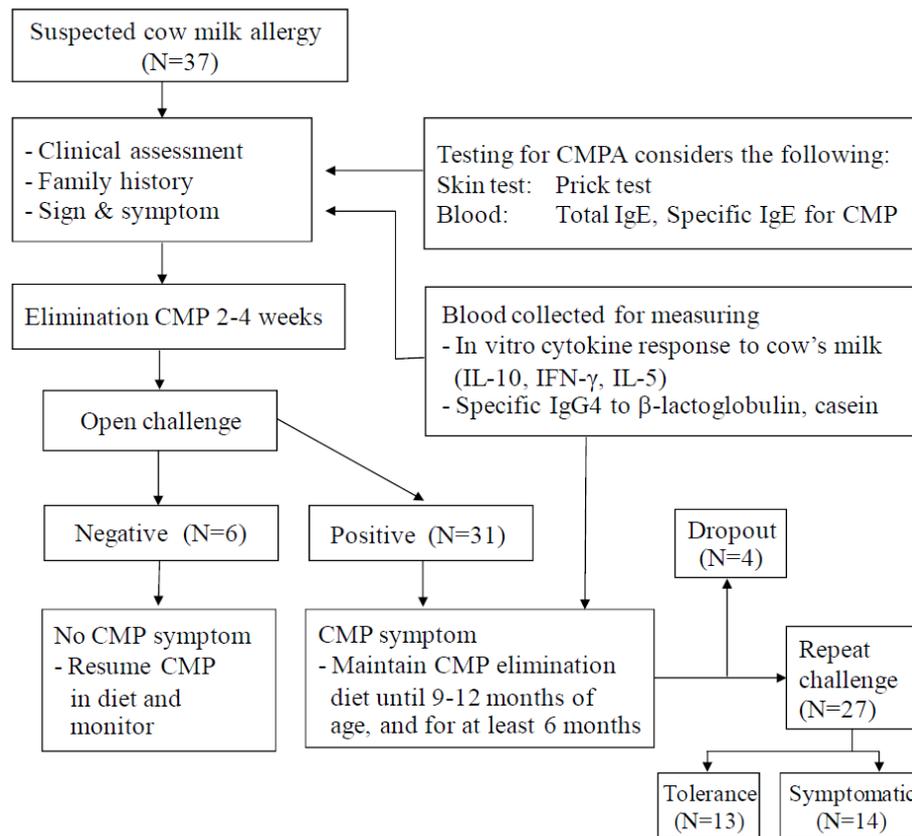


Figure 1. Diagnostic flow-chart

saline solution control. CMA was classified as IgE mediated if the CM-specific skin prick test response was positive.

Collection of samples

Venous blood samples were collected at two points in time: during symptomatic episodes and 6 months after elimination of cow's milk from the diet. Sera were stored at -20°C until analysis. PBMC were separated from whole blood and were stimulated with cow's milk for 3 day at 37°C in 5% CO_2 and the supernatants were collected and stored at -70°C until analysis.

Total and specific IgE levels

Serum levels of total and milk-specific IgE antibodies were analyzed with ImmunoCAP[®] (Phadia, Uppsala, Sweden) according to the recommendations of the manufacturer. The specific IgE to CM values were considered to indicate sensitization if the level was higher than 0.35 kUA/L.¹⁷

IgG4 antibody levels to food allergens

IgG4 antibodies to BLG and CAS in serum were analyzed with ImmunoCAP[®] (Phadia, Uppsala, Sweden) according to the recommendations of the

manufacturer. Then BLG and the CAS-specific IgG4/CM-specific IgE ratios were calculated.

In vitro peripheral blood mononuclear cells cytokines response to cow's milk protein

Isolation of PBMCs

Freshly drawn heparinized blood was kept at room temperature for not longer than 6 hours. Approximately 3 ml. of heparinized blood was collected. PBMCs were isolated by density gradient centrifugation using standard procedures. The culture medium used contained RPMI 1640 (Sigma Chemical Co.-Aldrich, Gillingham, United Kingdom), 50 U/ml penicillin, 50 g/ml streptomycin, 2 mM L-glutamine and 50 ml heat inactivated BSA.

Incubation of PBMCs

PBMCs were poured into 50 ml of PBS in a tube and spun in a centrifuge at room temperature for 15 min at 2000 RPM. The supernatant was decanted and PBMCs added at 1×10^6 cells per well in triplicate for each stimulus. Cells were incubated for 72 hours in a 5% CO_2 -humidified atmosphere, at 37°C in the absence or presence of respective antigens or mitogens: BLG (500 mcg/ml) and phytohaemagglutinin (PHA; 10 mcg/ml; Sigma Chemical Co.-Aldrich).



The cells were then counted to determine the growth in cell numbers following the reaction to the stimulating agent. The supernatant was stored at a temperature -70°C .

Detection of in vitro PBMCs cytokine response to cow's milk protein

The level of cytokine produced by activated PBMCs was examined. Instant ELISA (Bender Med System, Vienna, Austria) was used to capture and detect monoclonal antibodies to IL-10, IFN- γ and IL-5.

Ethical approval

The study was approved by the Research Ethical Committee of Faculty of Medicine Ramathibodi Hospital, Mahidol University. Written informed consent was obtained from the parents in each case. To minimize the discomfort for children, topical analgesic cream was used prior to the collection of blood samples.

Statistic analysis

Demographics data, cytokine levels (IL-10, IFN- γ , IL-5), specific IgE to CM levels, total IgE levels, specific IgG4 to BLG and CAS levels, specific IgG4 to BLG and CAS/IgE-CM ratios were subjected to descriptive analysis. To compare cytokine levels, specific IgG4 to BLG and CAS levels and BLG and CAS-specific IgG4/IgE-CM ratios between symptomatic patients and tolerant patients, the t-test was used for normally distributed data or, otherwise, a nonparametric test was used for abnormally distributed data. All statistics were analyzed using SPSS version 18.

Results

There were 27 subjects, 19 boys and 8 girls. The ages ranged from 1 to 42 months. The mean age at diagnosis CMPA was 8.1 months and mean age of onset of symptoms was 3 months. Patients with a previous history of immediate CMPA reactions reacted within 7 minutes (1 minute – 2 hours), whereas those with previous history of late reactions could take up to 14 days (4 days – 4 months) to react. The mean age of ceasing breast feeding only was 1.9 month (0 – 10), while that of starting CM formula was 1.5 month (0 – 10). Organ system involvements were skin (66.7%), gastrointestinal (GI) (44.4%) and respiratory system (66.7%). Eczema (37%) and maculopapula rashes (55.6%) were the most common manifestation of skin sensitization. Diarrhea (29.6%), vomiting (25.9%) and lower GI bleeding (22.2%) were the common GI manifestations. Hypersecretion (63%) and rhinitis (55.6%) were commonly found in those

with respiratory involvement. Other clinical manifestations found were anemia (44.4%), failure to thrive (22.2%) and eosinophilia (18.5%). In this study, patients were classified into three groups, namely IgE-mediated (N = 6), non IgE-mediated (N = 7) and mixed type (N = 14). The clinical manifestations of IgE-mediated allergy were immediate reactions (urticarial rash), specific IgE for CM more than 2 kUA/L and the CM oral challenge test produced clinical manifestations such as urticaria, angioedema and wheezing (no anaphylaxis). Non IgE-mediated and mixed type allergies were characterized by clinical manifestation such as skin manifestation (eczema, atopic dermatitis), GI symptoms (LGIB, diarrhea, vomiting and UGIB) and respiratory symptoms (rhinitis and hypersecretion) as illustrated in Table 1. The treatment was to avoid CM and instead use extensive hydrolysate formula (59.3%), soy formula (37%), breast-feeding (29.6%), partial hydrolysate formula (7.4%) or amino acid formula. (Some patients use more than one formula) The proportion of children with a maternal history of atopic diseases was 40.7%. The mean volume of CM during pregnancy was 726 ml/day and during lactation 427 ml/day. Fifty-two percent of parents had atopic diseases. CMPA in siblings was found in 7.4% and other atopic diseases were found in 14.8%.

The IgE-sensitization was associated with specific IgE to CM of more than 0.35 kUA/L (N = 11) or SPTs positive to CM and/or fresh cow milk (N = 20). Forty-eight percent ($n = 13$) of the patients could tolerate CM by 13.38 months (8 – 19 months). The mean level of specific-IgE to CM was 4.1 kUA/L (range 0.35 – 14.3 kUA/L). Based on our results, clinical manifestations, the level of specific IgE for CM, and SPTs cannot be used to predict clinical tolerance to CMP. Therefore, the re-challenge test is the best method to determine this.

In patients who tolerated CM, IL-10 cytokine responses to BLG were significantly increased during the tolerance period as compared to the symptomatic period (212.93 vs 142.46 pg/ml, $P = .011$) as depicted in Figure 2. There were no significant differences in IL-5 and IFN- γ cytokine responses to BLG during the two periods in the CM tolerant and CM intolerant groups (91.03 vs 46.78 pg/ml, $P = .317$ and 23.65 vs 22.32 pg/ml, $P = .375$) respectively.

In the CM tolerant group, there was a significant increase in IgG4 to BLG (33.89 vs 1.64 pg/ml, $P = .017$) and the BLG-IgG4/CM-IgE ratio (40.53 vs

Table 1. Clinical manifestations of IgE-mediated, non-IgE-mediated, and mixed type CMPA

1st sIgE CM	2nd sIgE CM	oral tolerance group	persistent allergy group	clinical manifestation	SPT
1. IgE-mediated					
2.03	1.71		Yes	atopic dermatitis, rhinitis, hypersecretion, vomiting	positive
2.63	2.91		Yes	atopic dermatitis, rhinitis, hypersecretion	positive
4.36	0.44	Yes		atopic dermatitis, rhinitis, hypersecretion, diarrhea,	positive
8.42	21.2		Yes	urticaria	positive
10	4.41		Yes	urticaria	positive
14.3	4.07	Yes		urticaria, vomiting	positive
2. Non-IgE mediated					
< 0.35	< 0.35		Yes	diarrhea, LGIB, hypersecretion, rhinitis	Negative
			Yes, moderate persistent		
< 0.35	0.05		asthma	wheezing, hypersecretion, rhinitis, FTT	Negative
< 0.35	0.21	Yes		diarrhea, LGIB, hypersecretion	Negative
< 0.35	< 0.35		Yes	eczema	Negative
< 0.35	< 0.35	Yes		hypersecretion, rhinitis	Negative
< 0.35	< 0.35		Yes	diarrhea, LGIB, hypersecretion, rhinitis	Negative
				eczema, rhinitis, hypersecretion, mild anemia,	
< 0.35	< 0.35	Yes		eosinophilia	Negative
3. Mixed type allergy					
0.47	< 0.35	Yes		eczema, hypersecretion, rhinitis	positive
0.61	0.64	Yes		diarrhea, LGIB	positive
0.92	0.94		Yes	atopic dermatitis, hypersecretion	positive
			Yes moderate persistent		
0.65	1.65		asthma	atopic dermatitis, hypersecretion, rhinitis, anemia, eosinophilia	positive
0.61	0.65	Yes		hypersecretion, rhinitis	positive
< 0.35	0.64	Yes		urticaria, vomiting	positive
< 0.35	0.55		Yes	diarrhea, LGIB, vomiting, FTT, anemia	positive
< 0.35	2.63		Yes	eczema, hypersecretion, rhinitis, anemia	positive
< 0.35	2.73		Yes	atopic dermatitis, hypersecretion, rhinitis	positive
< 0.35	1.18		Yes	eczema, hypersecretion, rhinitis	positive
< 0.35	0.07	Yes		eczema, rhinitis, FTT, mild anemia	positive
< 0.35	< 0.35	Yes		diarrhea, LGIB, anemia	positive
				eczema, rhinitis, hypersecretion, mild anemia,	
< 0.35	< 0.35	Yes		eosinophilia	positive
< 0.35	< 0.35	Yes		eczema, rhinitis, hypersecretion, vomiting, UGIB	positive

1.42, $P = .004$), but not in IgG4 to CAS (10.36 vs 1.86 pg/ml, $P = .07$) and the CAS-IgG4/CM-IgE ratio (10.33 vs 1.04, $P = .069$), when compared to the symptomatic period, as shown in Figure 3 and Figure 4. Furthermore, IgG4 to BLG and the BLG-IgG4/CM-IgE ratio were increased in IgE-mediated and mixed type CMPA in the tolerant group.

Discussion

Cow's milk protein allergy in infants is often transient and resolves when they grow up, with a minority remaining symptomatic. Children with

persistent CMPA are characterized by a significantly increased production of the Th2 cytokines IL-4 and IL-13. In contrast, the cow milk protein-specific production of IFN- γ is low in transiently allergic infants compared with control subjects.¹⁸ Tiemessen MM, et al.¹⁹ found activated T-cell clones to cow's milk in tolerant control subjects produced IL-10 but less IFN- γ . These findings suggest that activated CD4+ T cells (characterized by a high CD25 expression) might contribute to the tolerogenic immune response toward an antigen such as cow's milk, through the production of IL-10.

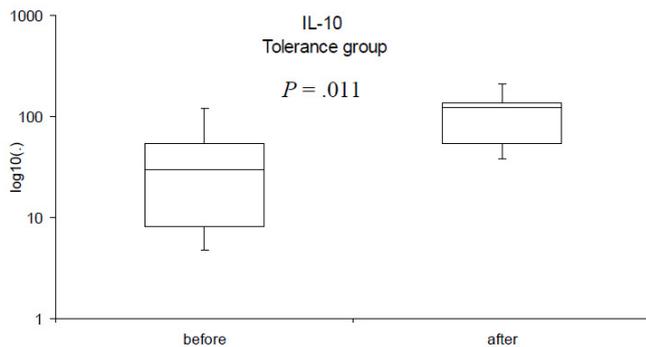
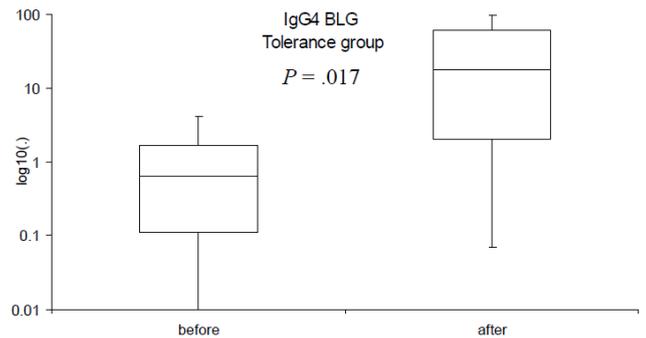


Figure 2. *In vitro* cytokines responses to β -lactoglobulin : IL-10

IL-10 is a major regulatory cytokine, which plays an important role in the induction and maintenance of anergic states. High IL-10 levels were found in patients who were found to be food tolerant, having been previously food-allergic.^{9,11} From this study, the *in vitro* cytokines response to BLG were determined during CM tolerant and symptomatic periods. The IL-10 levels of tolerant group were significantly increased (212.93 vs 142.46 pg/ml, $P = .011$). However, IFN- γ and IL-5 levels were not different between the two periods. These data indicate an important role for IL-10 secreting cells in the development of tolerance. It should be pointed out that the IFN- γ level in the tolerant period is in fact higher than that in the symptomatic period, but the difference is not statistically significant. The reason that the IFN- γ levels are high might be the result of a Th1-skewed response.

Several studies have shown that IgG4 to specific-food allergen and the IgG4/IgE ratio increase in tolerant patients.^{15,16} In the present study, we found that the specific IgG4 to BLG level increased during the symptomatic period in the CM tolerant group. Specific IgG4 to CAS levels and CAS-specific IgG4/IgE-CM ratios also increased but not significantly. Furthermore, the BLG-specific IgG4/IgE-CM ratios of the tolerant group increased significantly during the symptomatic period. High levels of CM-specific IgG4 and the IgG4/IgE ratios were associated with tolerance to CM in CMPA patients who had outgrown their allergy. The immunological response associated with the development of tolerance appears to be secondary to a modified Th2 immune response that includes high levels of IgG4 antibodies in combination with low levels of IgE antibodies. However, the *in vitro* cytokine response to CM, IL-10, which is secreted

A. Specific IgG4 to β -lactoglobulin



B. Specific IgG4 to casein

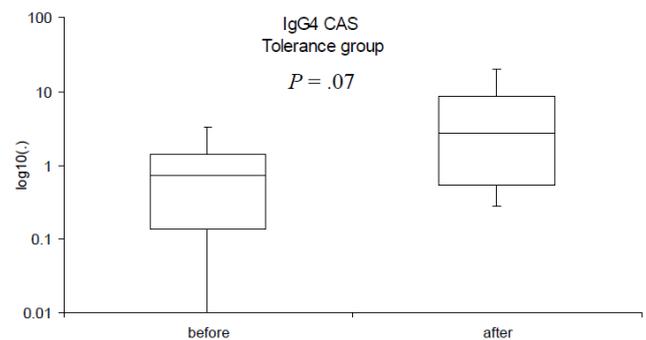


Figure 3. Specific IgG4 to β -lactoglobulin and casein

by regulatory T cells, suppresses IgE production, and simultaneously increases IgG4 production.

In conclusion, in our study of 27 subjects we found that IL-10, specific IgG4 to BLG and the BLG-specific IgG4/IgE ratio were higher in CMPA patients tolerant of CM than in those with episodes of symptoms. In fact, IgE-mediated and mixed type mechanisms related to CMPA can be used to calculate the IgG4/IgE ratio. Thus, it can be implied that specific IgG4 to BLG and BLG-specific IgG4/IgE-CM ratios may be useful for CM re-challenge, when considering together with clinical manifestations and the result of measurements of specific IgE to CM and SPTs. It should be pointed that the small sample size in this study limits the usefulness of this conclusion.

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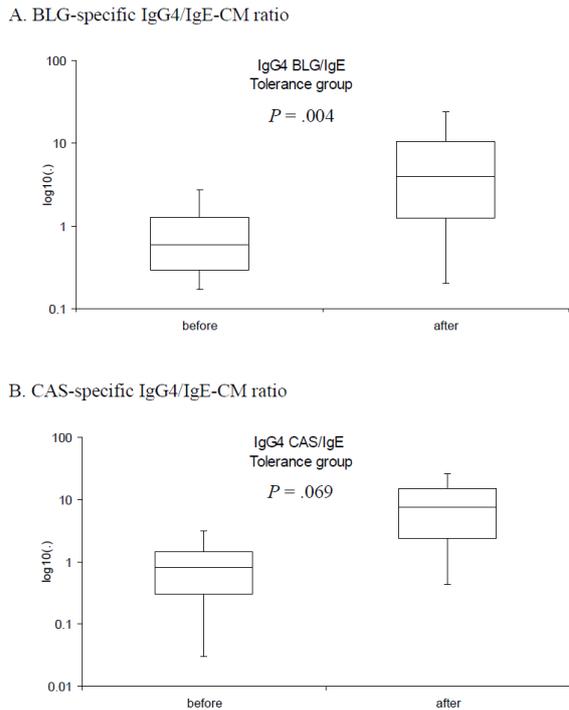


Figure 4. BLG and CAS-specific IgG4/IgE-CM ratios

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