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Cow's milk stimulated lymphocyte proliferation and TNF α secretion in hypersensitivity to cow's milk protein

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Abstract

Although there is no reliable single laboratory test available for the diagnosis of cow's milk allergy, if an allergic mechanism is suspected, a number of laboratory studies may be useful in delineating specific proteins responsible for these disorders. In the current study we analyzed in vitro lymphocyte proliferation assays, specific secretion of TNF α in supernatant cultures and specific IgE, IgG, and IgA in a group of patients with hypersensitivity to cow's milk antigens. The stimulation index against a cow's milk antigen mixture, α -lactalbumin, β -lactoglobulin, and casein was significant higher in the group of patients maintained on cow's milk-free diet for less than 4 months compared with the values observed in the control group and in the group of patients without a close contact to cow's milk proteins. A significant increase in TNF- α secretion was observed in supernatants from patients with close contact to cow's milk (CM). Specific IgE was detected in 59.3% of the patients, with higher specific IgE levels in patients who were not positive for the proliferation assay, suggesting a clear difference in the two mechanisms proposed as effectors in this disease. No differences in specific IgG and IgA levels were observed between the patient group and the control group, with a great dispersion among individuals in all groups tested. We conclude that a combination of the assays tested in this study, such as proliferative assay of peripheral blood mononuclear cells to CM, the quantitation of TNF α in culture supernatants, and serum specific IgE determination, are useful laboratory tests to identify cow's milk allergy among patients with immediate and nonimmediate adverse reactions, reducing the need for food allergen challenges in young children.

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Introduction

Cow's milk allergy (CMA) is defined as an immunologically mediated reaction against cow's milk antigens [1]. The incidence of CMA ranges from 1.9 to 7.5% in population-based studies [2]. Symptoms appear during the first year of life and in almost all patients within the first month of their life [3]. They occur within days or weeks after commencing feeding with a cow's milk-

based formula or already at their first exposure [3]. CMA can be presented with a wide spectrum of clinical reactions, including cutaneous, gastrointestinal, and respiratory symptoms as well as systemic anaphylactic symptoms [4]. Clinical symptoms involve immediate or delayed reactions, operating separately or together [5]. Immediate reactions are mainly IgE dependent, leading to cutaneous, intestinal, or respiratory symptoms and in some cases to anaphylactic reaction [6]. Delayed reactions most often imply T cell-dependent mechanisms and can be operative both at the skin and at the intestinal level [6].

The diagnosis of CMA is based on a definite disappearance of symptoms of CMA after elimination of cow's milk from the diet (and the breastfeeding mother's diet)

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[7], reoccurrence of identical symptoms during cow's milk challenge, disappearance of symptoms after reelimination of cow's milk, and exclusion of lactose intolerance and coincidental infections [8]. A double-blind placebo-controlled food challenge is considered as the preferred diagnostic tool in confirming food allergy in children [9].

Although there is no reliable single laboratory test available for the diagnosis of CMA, if an allergic mechanism is suspected, a number of laboratory studies may be useful in delineating specific proteins responsible for IgE-mediated disorders [10]. Indeed, the prick/puncture skin test (PST) and the Radioallergosorbent test (RAST) provide useful methods for establishing whether a patient possesses IgE antibody to cow's milk antigen [11]. These tests indicate the presence of allergen-specific IgE, but they do not establish the diagnosis of clinical food allergy [8]. Whereas cow's milk-specific IgE and PST assesses only CMA with an IgE-mediated mechanism, there are fewer tests available for detecting delayed hypersensitivity, the mechanism of which is not fully understood. A positive patch test reaction has been associated with delayed-type reactions [11], and it has also been reported to correlate with the immediate-type reaction [12]. Various tests on cellular immunity have been reported; especially because T cell-mediated reaction is mainly considered to play a role in delayed gastrointestinal reactions to cow's milk proteins [13–15]. Despite disparities in results from analyses of cellular responses to cow's milk antigens [13–16], some investigators have proposed that antigen-stimulated lymphocyte proliferation assays should be used as a diagnostic or predictive test for food allergy.

In the current study we analyzed *in vitro* lymphocyte proliferation assay, specific secretion of TNF α in culture supernatants, and specific IgE, IgG, and IgA in a group of patients with adverse reactions to cow's milk antigens. We conclude that a combination of the following assays: proliferative response of peripheral blood mononuclear cells (PBMC) to cow's milk (CM) assay, the quantitation of TNF α in culture supernatants, and serum-specific IgE determination, are useful laboratory tests to identify cow's milk allergy among patients with immediate and nonimmediate adverse reactions, reducing the need for food allergen challenges in young children.

Materials and methods

Subjects

Forty-one children were included in the study. Patients were selected from Pediatric Clinic "Clinica del Niño" (Córdoba, Argentina). The patient group consisted of 27 children suspected of having allergy to cow's milk proteins on the basis of favorable response to the elimination diet and clinical relapse on challenge. The patients had

signs and symptoms of allergy after milk ingestion, such as morbilliform rash, urticaria, pruritus, angioedema, vomiting, gastroesophageal reflux, colic, diarrhea, laryngeal edema, protein losing enteropathy, failure to thrive, chronic abdominal pain, or wheezing. Symptoms resolved completely within 72 h of eliminating cow's milk proteins from the diet (e.g., cow's milk or any other milk-containing product). Some patients had eliminated milk proteins several months before they were included in the study. Milk allergy was verified on the basis of favorable response to the elimination diet and clinical relapse on challenge with cow's milk. Subjects in our study ranged in age from 1 month to 7 years. The control group comprised 14 nonatopic healthy children (ages ranging from 1 to 7 years) who had no clinical evidence of milk allergy or any food allergy.

Blood samples (2–5 ml) were collected and the lymphoproliferative assay was performed in a blind manner. Informed consent for inclusion in the study was obtained from all parents.

Lymphocyte proliferation assay

PBMC were isolated from heparinized blood by gradient centrifugation in Ficoll–Hypaque (Sigma). Peripheral blood mononuclear cells were suspended in culture medium consisting in RPMI 1640 supplemented with glutamax, sodium pyruvate, nonessential amino acids, Hepes buffer, penicillin/streptomycin, 50 mM 2-ME, and 10% FCS (Sigma). Peripheral blood mononuclear cells were distributed at a concentration of 1.5×10^5 cells per well in 0.2 ml volume, into flat-bottomed 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ) with or without different cow's milk proteins: α -lactalbumin (600 μ g/ml), β -lactoglobulin (600 μ g/ml), casein (600 μ g/ml), and an equimolar mixture of these proteins that we call cow's milk antigen mixture (CMP) (600 μ g/ml). All cell combinations were set up in triplicate. Plates were incubated for 5 days at 37°C in water saturated containing 7.5% CO₂ atmosphere. Subsequently, as a positive control of proliferation, the PBMC were incubated with the mitogen Concanavalin A (Sigma). DNA synthesis was measured by adding 1 μ Ci of [methyl-³H]thymidine ([³H]TdR) per well 18 h before harvesting the cells onto glass fiber filters. Labeled material was automatically harvested and counted in a β -plate scanner (Pharmacia, France). The response was expressed as a stimulation index (SI) calculated from cpm incorporated in antigen-pulsed cultures/cpm incorporated in cultures with medium. The cut-off levels were calculated with the mean values for proliferation indexes observed in the healthy normal children plus 2 SD. They were 1.60 ($0.92 \pm 2 \times 0.34$) for CPM, 1.74 ($1.05 \pm 2 \times 0.34$) for α -lactalbumin, 1.80 ($1.04 \pm 2 \times 0.38$) for β -lactoglobulin, and 1.97 ($1.13 \pm 2 \times 0.43$) for casein, respectively.

TNF- α quantitation in cell culture supernatants

TNF- α was assayed in the supernatants from the PBMC cultured in the presence of CMP as described above. One hundred microliters of culture supernatants was collected after 96 h of culture and TNF- α secretion was assayed by the use of a commercially available chemiluminescence immunometric assay kit (Immulite, DPC, Los Angeles, CA). The assay was performed according to the manufacturer's instructions in an automatic analyzer (Immulite, DPC). The TNF- α contents in culture supernatants were expressed in pg/ml, according to a standard curve ranging from 1.7 to 1000 pg/ml.

Determination of antibodies to cow's milk proteins

Protein antigen preparations were obtained from Sigma Chemical Co. (St Louis, MO). As antisera, rabbit anti-human IgG and IgA were used for specific IgG and IgA determination. ELISA was performed in 96-well microtiter plates (Maxisorp, Cambridge, MA) precoated with 50 μ l per well of 20 μ g/ml α -lactalbumin, β -lactoglobulin, or casein in 0.05 M carbonate buffer, pH 9.6. After overnight incubation at 4°C, microwells were washed three times with PBS–Tween and blocked with PBS/BSA 5% in PBS for 3 h at 37°C, rinsed with PBS–Tween 20 at 0.05%, and then filled with 50- μ l serum sample dilutions (1/25) and incubated for 1 h at 37°C. To detect specific IgG and IgA, plates were washed again and incubated with a rabbit anti-human IgG and IgA (Fc specific) horseradish-peroxidase-conjugated monoclonal antibody (Sigma Chemical Co) for 1 h at 37°C. The reaction was developed with *o*-phenyldiamine–H₂O₂. Absorbance was measured at 490 nm in a Titertek Multiskan.

For the detection of specific IgE in the human serum samples, we used a commercially available Specific IgE (ULEAST) Kit (Cypress Diagnostics, Leuven, Belgium). The assay was performed according to the manufacturer's instructions, which consisted in the incubation of serum sample dilutions with antigen-labeled dishes in a 96-well microtiter plate (Becton Dickinson) for 1 h at room temperature. After washing, the plate was incubated with a rabbit anti-human IgE (Fc specific) horseradish-peroxidase-conjugated monoclonal antibody, for 1 h at room temperature. The reaction was developed with TMB–H₂O₂. Absorbance was measured at 570 nm in a Titertek Multiskan. ULEAST results were scored by comparing them with reference sera: H (++++), A (++++), B (+++), C (++) and D (+). ULEAST scores of $\geq 1+$ were recorded as positive.

Statistics

LSD Fisher Alpha test was used for the comparison of the different groups.

Results

Proliferative response against cow's milk proteins

The proliferative responses of PBMC to CMP, α -lactalbumin, β -lactoglobulin, or casein in each patient with adverse reactions to cow's milk proteins and controls are shown in Table 1. No positive stimulation index was detected when we analyzed the response of PBMC from healthy nonatopic children to CMP, α -lactalbumin, β -lactoglobulin, or casein (Table 1). The SI cut-off levels in our assay were 1.60 for CPM, 1.74 for α -lactalbumin, 1.80 for β -lactoglobulin, and 1.97 for casein, respectively. Positive proliferative responses to one or more of the antigens tested could be detected in 15 of 27 patients (55.5%). As it can also be seen in Table 1, patients maintained on cow's milk-free diet for less than 4 months showed higher percentages of positivity in the lymphocyte proliferation assay, with 10 of 14 patients (71.4%) showing positive SI. We could observe that only 5 of 13 patients (38.5%) maintained on cow's milk-free diet for more than 4 months showed positive proliferative responses to one or more of the antigens assayed, suggesting that a close contact with the offending antigen is necessary to detect specific circulating PBMC (Table 1). Interestingly, most of the patients that showed elevated proliferative response to milk proteins have had gastrointestinal symptoms, such as diarrhea and colic, suggesting an association between the effector mechanism and the observed clinical manifestations. Fig. 1 shows a comparison of the SI of the control group, the SI of the total patients group, and the values observed when the patient group was divided in patients maintained on cow's milk-free diet for less or more than 4 months. When the values of SI against α -lactalbumin, β -lactoglobulin, and casein obtained for the total patient group were compared with the ones of the control group, a significant difference was observed only for CMP ($p < 0.05$). Stimulation indexes against CMP, α -lactalbumin, β -lactoglobulin, and casein were significantly higher in the patient group maintained on cow's milk-free diet for less than 4 months compared with the values observed in the control group and in the group of patients without a close contact to cow's milk proteins (Fig. 1).

No association between age levels and proliferative response was observed in the two subgroups of patients (Table 1), suggesting that age level is not a significant factor in the effector mechanism observed. As also shown in Table 1, not all patients were positive for all milk proteins assayed, supporting the importance of testing different milk proteins to improve the sensibility of the method.

TNF- α release in PBMC culture supernatants

As TNF- α is one of the mediators involved in adverse reactions to CMP and it is known that it directly alters the

Table 1
Clinical features, proliferative responses (SI), and specific IgE from patients and controls

Patient	Principal clinical manifestation	Age	Proliferation assay				Specific IgE		
			CPM	α -Lactalbumin	β -Lactoglobulin	Casein	α -Lactalbumin	β -Lactoglobulin	Casein
Normal children with no adverse reaction to cow's milk proteins									
N1	—	1y 2m	0.31	0.34	0.49	0.70	—	—	—
N2	—	1y 4m	0.96	1.07	1.42	1.33	—	—	—
N3	—	1y 6m	0.95	0.67	0.69	0.64	—	—	—
N4	—	1y	0.66	1.64	1.98	1.97	—	—	—
N5	—	2y	1.08	1.07	0.51	0.53	—	—	—
N6	—	2y	0.94	1.18	1.16	1.48	—	—	—
N7	—	3y	1.70	1.27	1.25	0.70	—	—	—
N8	—	3y	0.52	0.57	0.59	0.98	—	—	—
N9	—	3y 6m	0.95	0.95	1.12	1.59	—	—	—
N10	—	4y	1.03	1.60	0.80	1.00	—	—	—
N11	—	4y	0.80	1.20	1.20	0.90	—	—	—
N12	—	4y	0.85	1.09	1.05	1.35	—	—	—
N13	—	5y	1.40	1.10	1.20	0.92	—	—	—
N14	—	7y	0.77	1.02	1.04	1.74	—	—	—
X $\pm$ SD	—	2.96 $\pm$ 1.60	0.92 $\pm$ 0.34 (>1.60)*	1.05 $\pm$ 0.34 (>1.74)*	1.04 $\pm$ 0.38 (>1.80)*	1.13 $\pm$ 0.42 (>1.97)*			
Patients maintained on cow's milk-free diet for 4 months or more									
P1	DE	10m	0.80	0.85	0.99	0.91	—	+	—
P2	DI	10m	1.53	0.90	4.04	0.97	—	+	++
P3	PLE	1y 8m	0.50	0.60	0.40	0.30	++	+	+
P4	FT	1y 8m	1.03	0.88	1.56	2.98	+	++	++
P5	CO	2y 6m	1.03	2.25	0.97	1.52	—	—	—
P6	DI	4y	2.05	2.10	2.80	3.46	—	—	—
P7	FT	4y	0.70	0.85	0.96	0.50	—	+	ND
P8	DI	5y	0.72	1.08	0.65	0.69	+	—	++
P9	DE	5y	2.26	1.03	1.30	1.98	—	+	+
P10	DI	6y	0.86	1.05	2.24	1.95	—	—	—
P11	DI	7y	0.70	0.80	0.70	0.60	++++	++++	++++
P12	CAP	7y	0.71	1.05	0.90	0.28	—	+	+
P13	DE	5y	1.00	1.24	1.60	1.03	+	—	—
X $\pm$ SD		3.88 $\pm$ 2.20	1.07 $\pm$ 0.54	1.13 $\pm$ 0.49	1.47 $\pm$ 1.02	1.32 $\pm$ 1.01			
Patients maintained on cow's milk-free diet for less than 4 months									
P14	DI	11m	0.45	0.30	0.30	1.50	+	+	+++
P15	DI	11m	4.14	3.24	4.39	4.03	—	—	+
P16	DI	13m	0.64	0.66	0.52	0.28	—	—	—
P17	DI	1y 1m	1.15	1.68	1.30	0.74	+	+	+
P18	DI	1y 3m	1.57	3.12	0.95	1.41	—	—	—
P19	DI	1y 8m	2.17	2.82	3.28	4.71	—	—	—
P20	DI	3y 10m	0.86	1.06	1.42	1.22	+	—	++
P21	DI	4y	0.30	0.35	0.14	2.00	+	++++	—
P22	DI	4y	2.67	2.98	2.19	2.97	—	—	—
P23	DI	5y	2.05	0.50	1.20	0.60	—	—	—
P24	DI	6y	1.65	2.50	1.51	9.60	+	—	+
P25	DE	7y	2.14	1.70	2.40	0.91	—	—	—
P26	DI	7y	4.81	5.41	7.11	3.55	—	—	—
P27	DI	7y	3.33	2.56	2.35	2.13	—	—	—
X $\pm$ SD		3.62 $\pm$ 2.46	2.00 $\pm$ 1.36	2.09 $\pm$ 1.50	2.08 $\pm$ 1.86	2.55 $\pm$ 2.44			

Note. Values in parentheses are cut-off levels calculated with the mean values of SI + 2SD. PLE, protein losing enteropathy; FT, failure to thrive; CAP, chronic abdominal pain; DE, dermatitis; CO, colic; DI, diarrhea.

intestinal epithelial barrier, we assayed TNF- α secretion in the supernatants of the PBMC cultured with CMP from the two groups of patients and controls. As shown in Fig. 2, a significant statistical increase in TNF- α secretion is observed in supernatants from patients maintained on cow's milk-free diet for less than 4 months (close contact to the offending antigen) compared to the other patient

group (cow's milk-free diet for more than 4 months) and controls ($p < 0.05$).

Specific IgE, IgA, and IgG against cow's milk proteins

We also analyzed the IgE serum levels against α -lactalbumin, β -lactoglobulin, and casein. Table 1 shows that 16

PMBC proliferation against Cow's milk proteins

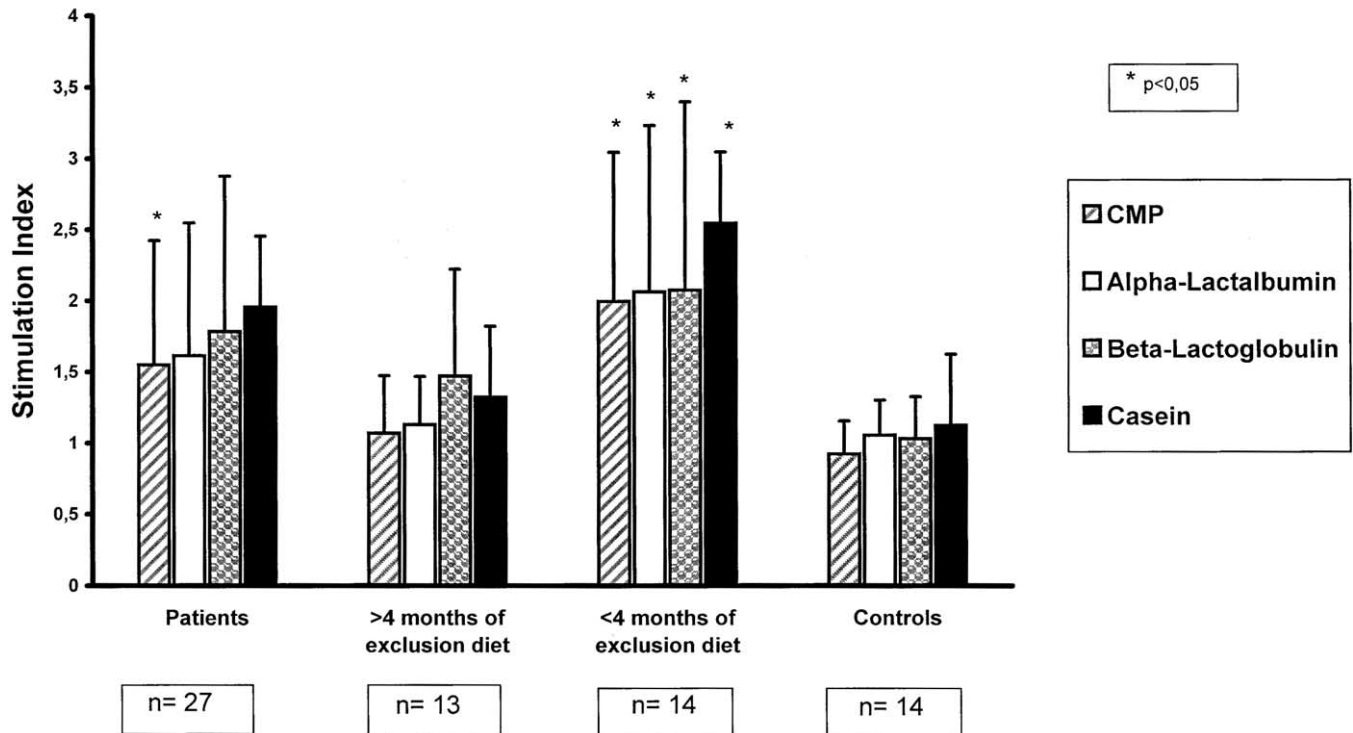


Fig. 1. Lymphocyte proliferation in response to CMP, α -lactalbumin, β -lactoglobulin, and casein stimulation in control subjects and patients with adverse reactions to cow's milk proteins. Patient group was divided in patients maintained on cow's milk-free diet for less or more than 4 months. * $p < 0.05$ vs control group.

of 27 patients (59.3%) had elevated levels of specific IgE against one or more antigens assayed. Interestingly, the highest IgE humoral response was observed in patients who were not positive for the proliferation assay. Only 5 patients (18.5%) showed positive responses to both methods, proliferative response and specific IgE assay. Only one patient was negative for both methods assayed.

We also analyzed the presence of specific IgG and IgA to cow's milk proteins. As is shown in Fig. 3A, serum IgG and IgA levels against β -lactoglobulin showed a considerable overlap among the values observed in the patient group and those observed in the control group. Similar results were observed when we tested serum IgG and IgA levels against α -lactalbumin (Fig. 3B) and casein (Fig. 3C). No differences were found between the levels of IgG and IgA to the antigens tested in control versus patient groups.

Discussion

The principle of elimination and challenge with the offending antigen has been the gold standard in the diagnosis of food allergy with predominantly gastrointestinal symptoms. However, the reintroduction of the antigen is poten-

tially dangerous. Consequently, there is a great need for predictive tests to overcome these drawbacks.

Although there is no reliable single laboratory test available for the diagnosis of CMA, if an allergic mechanism is suspected, a number of laboratory studies may be useful in delineating specific proteins responsible for these disorders [10–15]. In immediate reactions, laboratory studies and in vivo tests that measure circulating antigen-specific IgE and tissue-specific mast cell degranulation are useful tools in the diagnosis. Since specific IgE antibodies do not mediate about half of the reactions following the ingestion of milk [16], the study of cellular responses is very important. In late reactions various tests on cellular immunity have been reported but with disparities in their results.

Tainio and Savilahti [17] used a β -lactoglobulin lymphocyte stimulation test and they found, among young infants with immediate reactions to cow's milk proteins, that most did not have specific IgE to cow's milk proteins but they had positive results in the β -lactoglobulin stimulation test. In another study, Soumalainen et al. [15] measured the cellular immune response to cow's milk protein in patients with challenge-proven CMA manifested with either gastrointestinal or skin symptoms. They found that the SI for lymphocytes in patients manifesting CMA with gastrointes-

TNF-Alpha in culture supernatants from PBMC stimulated with CMP

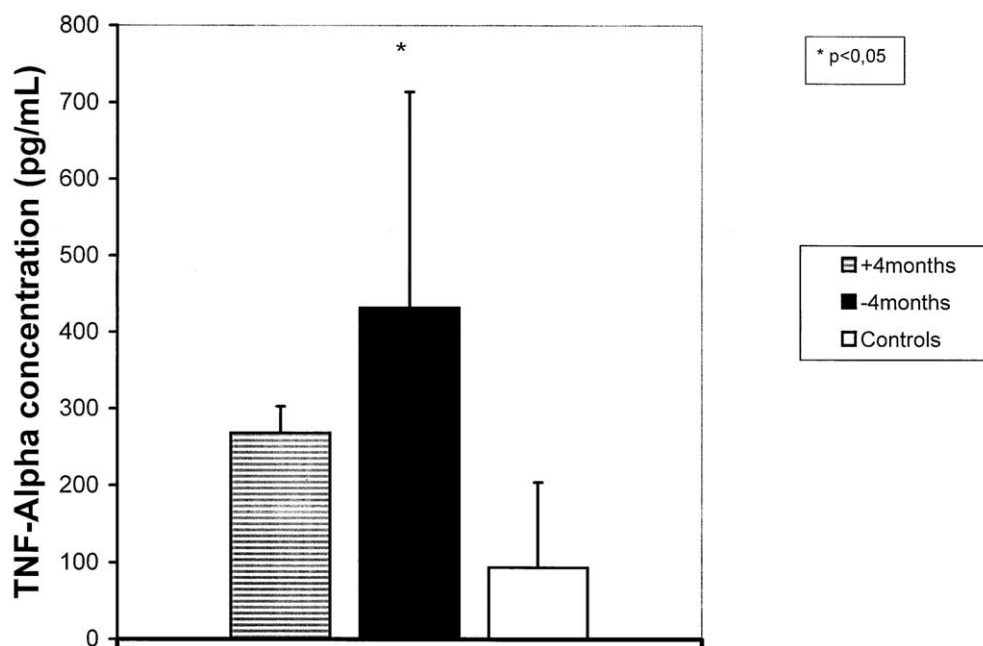


Fig. 2. TNF- α production in culture supernatants of PBMC stimulated with CMP. PBMC were stimulated with 600 μ g/ml of an equimolar mixture of cow's milk antigens (α -lactalbumin, β -lactoglobulin, and casein) for 5 days. Supernatants obtained were assayed using a chemiluminescence immunometric assay kit. * $p < 0.05$ vs control group.

tinal symptoms was significantly higher than that in patients with skin symptoms or patients with negative clinical challenge. Papadopoulos et al. [18] evaluated lymphocyte proliferation to β -lactoglobulin by quantitation of the proliferating cell nuclear antigen (PCNA) expression in PBMC, by flow cytometry. They showed a marked difference of PCNA expression between cow's milk-fed allergic infants and healthy controls and suggested that PCNA could be a useful marker in the assessment of lymphocyte proliferation to cow's milk antigens. Moreover they demonstrated that low cow's milk antigens diets are related with reduced lymphocyte reactivity. Kondo et al. [19] demonstrated that proliferative responses of PBMC to different food antigens in nonimmediate type hypersensitivity of food allergy are useful for the detection of allergens. However, Hoffman et al. [20] indicate that lymphocyte proliferation assays are neither diagnostic nor predictive of clinical reactivity in individual patients with milk allergy. They found that lymphocytes of many control patients are highly responsive to milk antigens and lymphocytes of many patients with milk allergy are not in the experimental conditions used in their studies.

In the present report, in agreement with several other reports that proposed that tests on cellular immunity are useful for the detection of allergens in nonimmediate types of food allergy, we showed that the SIs against CMP, α -lactalbumin, β -lactoglobulin, and casein were higher in the patient group compared with the values observed in the

control group. Moreover, the stimulation indexes against CMP, α -lactalbumin, β -lactoglobulin, and casein were significantly higher in the patient group maintained on cow's milk-free diet for less than 4 months compared with the values observed in the control group and in the group of patients without close contact to cow's milk proteins. The absence of response in the group of patients without close contact to cow's milk proteins could be attributed to the loss of circulating specific PBMC, may be recruited to the affected tissue in the sensitized children, or perhaps the hypersensitivity to cow's milk protein in these patients could be mediated by other mechanisms (such as IgE). It is known that the prick/puncture skin test and the RAST provide useful methods for establishing whether the patient possesses IgE antibodies to specific foods, although they do not establish the diagnosis of clinical food allergy. In the same way, the measurement of cellular immune response to cow's milk protein by lymphocyte proliferative assay would not establish the diagnosis of clinical food allergy but could be useful to ascertain whether the patient possesses sensitized T cells to specific foods.

It is largely known that specific antigen induces clonal expansion of T cells and the differentiation of its progeny into armed effector T cells able to synthesize all the proteins required for their specialized functions [21]. Several reports demonstrate that the measurement of cytokines in the culture supernatants may also be useful for the diagnosis of CMA [22,23]. Indeed, mononuclear cells from infants al-

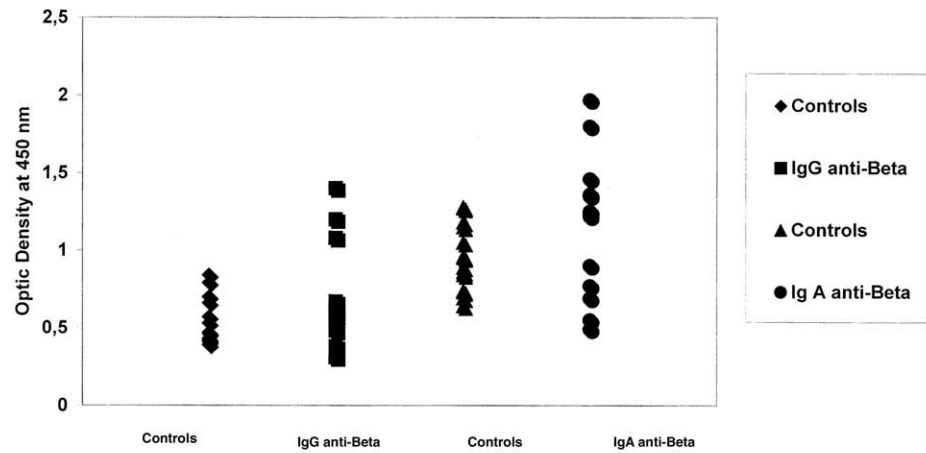
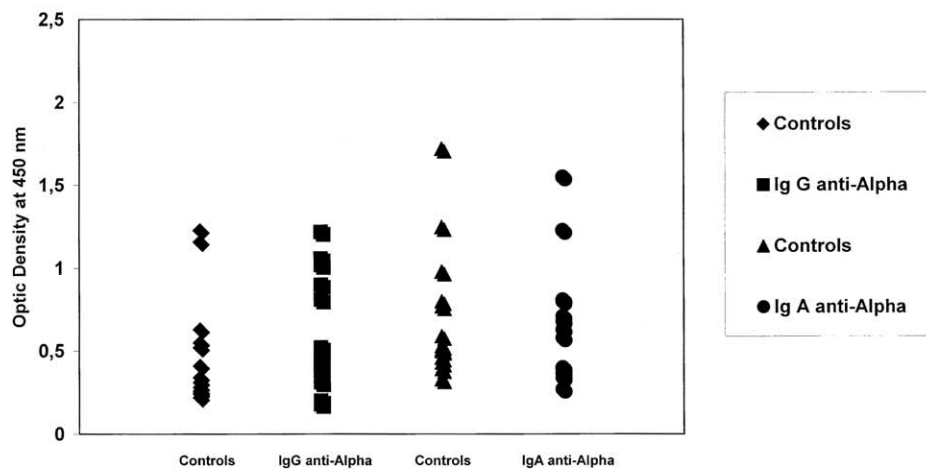
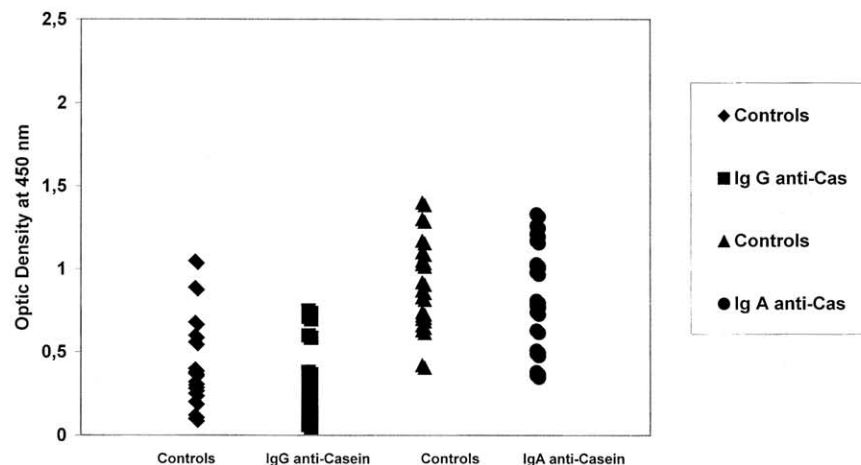
A Serum IgG and IgA against Beta-lactoglobulin**B Serum IgG and IgA against Alpha-lactalbumin****C Serum IgG and IgA against Casein**

Fig. 3. Serum levels of IgG and IgA against β -lactoglobulin (A), α -lactalbumin (B), and casein (C). Plates were precoated with 20 μ g/ml of β -lactoglobulin, α -lactalbumin, or casein and incubated with an appropriate dilution of serum. To detect IgG and IgA, horseradish peroxidase-conjugated antisera were used.

lergic to cow's milk secrete TNF- α and they propose that, during CMA, the higher level of TNF- α released by PBMC after milk protein challenge acts synergistically with INF- γ to increase the intestinal permeability [24]. It was also shown that intact rather than intestinally processed proteins stimulate PBMC to release TNF- α and alter intestinal barrier ability [25]. Moreover, it has been shown that the pattern of TNF- α secretion in response to cow's milk proteins is different in CMA infants with cutaneous or digestive symptoms and it has been suggested that TNF- α release might predict clinical relapse on challenge [26]. In the present report we observed a significant increase in the TNF- α secretion in supernatants of cultures from patients with close contact to cow's milk proteins compared to the other patient group maintained on cow's milk-free diet for more than 4 months and controls. It is interesting to note a correlation between elevated proliferative response and TNF- α secretion with gastrointestinal symptoms, such as diarrhea and colic, suggesting an association between the effector mechanisms and the observed clinical manifestations.

In agreement with results reported by several authors [8], IgE serum levels against α -lactalbumin, β -lactoglobulin, and casein were present in only 59.3% of the patients included in our study. Interestingly, the highest IgE humoral response was observed in patients who were not positive for the proliferation assay, suggesting a difference in the two mechanisms proposed as effectors in this disease (hypersensitivities type I or IgE mediated and type IV or cellular mediated, respectively). Only 18.5% of the patients showed positive responses to both methods, proliferative response and specific IgE assay. Only one patient was negative for both methods assayed. Interestingly, 37% of the patients in our study showed elevated proliferative response against one or more antigens tested and not elevated specific IgE levels, showing the importance of using a combination of tests that measure IgE and cell-mediated mechanisms when hypersensitivity to CM is suspected.

Food antigen-specific IgG antibodies are generally elevated in patients with food allergy affecting the gastrointestinal tract [8] but their specificities typically reflect the type of foods ingested and are not indicative of specific food-related pathogenesis [27,28]. May et al. [28] showed that elevated levels of antibodies to milk proteins in sera, characteristic of infants fed with cow milk, were found to decline with age, so that sera from children who were 6 to 15 years of age, not hypersensitive to food, had significantly lower levels than the infants. In contrast, sera from age-matched children, who were shown to have hypersensitivity to some food, were found to have levels of antibodies to milk proteins as elevated as in infancy. Host et al. [29] studied IgG, IgG subclass, and IgE antibodies to dietary proteins in infants with cow's milk allergy. No differences were found between the levels of IgG and IgG subclass antibodies to β -lactoglobulin in control versus infants with CMA. In another study the concentration of serum immu-

noglobulin levels to different cow's milk proteins in patients with CMA was assayed [30]. The authors found a close correlation between all individual IgA and IgG antibodies to different cow's milk proteins but no association between the clinical manifestations and the IgG and IgA antibody levels to the cow's milk proteins. Since a considerable overlap among the values observed in the different groups of patients was found in this study, they proposed that there is a limitation of these tests for predicting the prognosis. In our study IgG and IgA levels against cow's milk proteins also showed a considerable overlap among the values observed in the patient group and the ones observed in the control group.

We propose that a combination of the proliferative responses of PBMC to milk antigens, the presence of TNF α in culture supernatants, and specific IgE are useful laboratory tests in identifying cow's milk allergy among patients with various manifestations (IgE and cell-mediated), reducing the need for food allergen challenges in young children.

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