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The aim of this study was to compare the allergenicity of goat milk with cow milk in an animal model of food allergy. Either goat or cow milk was introduced as the first source of foreign protein following weaning of the mice from their mothers.

Milk plus an adjuvant compound (cholera toxin) was given to 3 week old mice immediately after weaning. The adjuvant compound forced the mice to become sensitized to the milk. Mice were given a total of six doses of goat or cow milk with the adjuvant to mimic the development of food allergy. At the end of the sensitization period, mice were challenged with either goat or cow milk and the allergic response was evaluated.

Eight of 13 mice sensitized with cow milk had diarrhea, compared with only 1 out of 13 mice sensitized with goat milk.

Allergic reactions in mice challenged with cow milk were almost 3 times greater compared to mice challenged with goat milk.

Plasma histamine levels concentrations in mice challenged with cow milk were over 6 times higher than mice challenged with goat milk.

These responses are consistent with goat milk being much less allergenic than cow milk when introduced into the diet of the mice after weaning.

This study adds critical support to our hypothesis that use of Goat Milk Infant Formula as the sole milk feed following breast feeding will be associated with lower prevalence or delayed onset of food allergy-related symptoms than infants fed cow milk infant formula.

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Goat Milk is Less Immunogenic than Cow Milk in a Murine Model of Atopy

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ABSTRACT
Objectives: Cow milk protein allergy occurs in 2% to 6% of infants population. Goat milk has been used as an alternative to cow milk, but there is probably some cross-reactivity between the milks. Little is known about the allergenicity of goat milk *per se*. The aim of this study is to compare cow and goat milk allergenicity in a mouse atopy model.
Methods: Balb/C mice were intragastrically sensitized to cow or goat milk by five doses administrated weekly. Six weeks after the first dose mice were killed, sera were collected and spleens removed for analysis.
Results: The number of mice with diarrhea was significantly higher in the cow milk-sensitized group than in the goat milk-sensitized group. Serum cow milk-specific immunoglobulin G1 and histamine levels were also significantly higher in cow milk-sensitized mice. Cytokine production by spleen derived T cells showed a Th2 response with high levels of interleukin-4 production and low levels of interferon-γ in cow milk-sensitized mice. In addition, goat milk induced a lower lymphocyte sensitization as a result of a significant decrease in the specific proliferation ratio of these cells.
Conclusion: Goat milk, when used as the first source of protein after a breast-feeding period, is less allergenic than cow milk in mice. Further studies are needed to clarify if goat milk is suitable as an alternative to cow milk in milk based formulas for infant nutrition. ***JPGN 39:354–360, 2004.*** **Key Words:** Goat milk—Atopy—Cow milk allergy—Infant nutrition—Sensitization. © 2004 Lippincott Williams & Wilkins

INTRODUCTION

Food allergies occur in 5% to 10% of infants and young children (1,2). Cow milk protein allergy (CMPA) is the most common sensitivity of young infants, with a 2- 6% incidence (3,4). This atopic disease is associated with a broad spectrum of immunoglobulin (Ig) E-mediated reactions that are mostly expressed as immediate symptoms, such as urticaria, rhinoconjunctivitis, asthma, vomiting, diarrhea and sometimes systemic anaphylactic shock and death (5).

CMPA may not be an isolated phenomenon, but may herald the beginning of an "atopic career." Infants and children with CMPA may also develop other atopic diseases such as other food allergies, rhinitis, asthma or early atopic dermatitis, a condition affecting up to 15-20% of normal children (6–10).

Human milk contains numerous factors protective against atopic disorders (11,12). However, breast-feeding is not always possible and in most cases children receive breast milk for less than 6 months. At the end of the breast-feeding period, children are usually given a cow milk based formula and at this time some become sensitized to cow milk (CM) and develop immediate symptoms after ingestion of cow milk. For these children a milk substitute must be provided. The most common alternatives to cow milk are soy milk and hydrolyzed formulas. There is, however, evidence that 10% to 40% of children allergic to CMP do not tolerate soy derivatives (13–15). Hydrolyzed formulas are usually tolerated by allergic children but some studies report that these formulas fail to induce a desired oral tolerance in rodents (16–18). In addition, they lack some of the bioactive peptides present in untreated cow milk that play important roles in immunomodulation (19).

The identification of a more suitable protein source for cow milk allergic children represents an important goal for pediatricians and nutritionists. Goat milk (GM) has been proposed as an alternative to cow milk because some allergic children can tolerate goat milk derivatives (20,21). However, several studies demonstrate that children with CMPA are also sensitized to goat milk protein, suggesting cross-reactivity between cow and goat milk proteins (22–24). There are rare reports of patients allergic to goat but not to cow milk proteins (25). These reports suggest that goat milk may not be a suitable protein source for children with CMPA.

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There is evidence, nevertheless, to believe that GM is less allergenic than CM because the content in α-casein, one of the most important antigens in CM, is lower in GM (26). It has been reported that α-casein acts as a carrier for other allergens in cow milk such as β-lactoglobulin, which is tightly linked to the casein micelles and therefore more difficult to digest (27). The lower content of α-casein in GM might allow a better digestion of β-lactoglobulin and other allergens. Therefore, GM could be less immunogenic when introduced into the diet of non-sensitized children. In this study we compared the allergenicity of CM and GM in a mouse model of orally induced atopy.

MATERIALS AND METHODS

Reagents and Milk Proteins

Cholera toxin (CT), concanavalin A (Con A) and lipopolysaccharide (LPS) were all purchased from Sigma Chemical Co. (St Louis, MO). Antibodies for ELISAs were purchased from Bethyl (Montgomery, TX) in the case of immunoglobulins or from Biosource (Camarillo, CA) for cytokines. All the other products were of the best grade available and purchased from Sigma. Deionized water, further purified with a Millipore Milli-Q (Bedford, MA) system, was used.

Homogenized CM was obtained from Puleva Food S.A. (Granada, Spain) (protein composition in g/liter: total protein: 36, total casein 29.5, α_{s1} casein: 11.9, α_{s2} casein: 3.1, β casein: 9.8) whereas GM was obtained fresh from murciano-granadina dairy goats in a local farm (protein composition in g/L: total protein: 33, total casein 28, α_{s1} casein: 1.4, α_{s2} casein: 7, β casein: 18.9) and submitted to the same UHT treatment as CM. Whole milk thus obtained was centrifuged at 3500 rpm for 10 minutes at 4°C and the upper layer of fat was discarded to obtain a skimmed milk. The content of protein in CM and GM was measured by the Kjeldhal method as previously described (28). Both milks were stored at −20°C in 1-mL aliquots and defrosted immediately before use in experiments.

Animals

Just weaned, 3 week old female Balb/C mice were purchased from the Granada University breeding colony and housed in a light controlled environment (12 hour cycle of light/dark) at 22°C. Animals (13 per group) were maintained on a regular mouse chow diet under pathogen-free conditions. Guidelines for the care and use of animals were followed as described (29). The entire experiment was conducted three times with similar results.

Sensitization and Challenge by Oral Administration of Antigen

Mice were sensitized intragastrically with CM or GM plus CT as an adjuvant and boosted five times at weekly intervals. Intragastric feeding was performed by means of a PVC tube feeding needle purchased from Vygon (Ecoue, France). Mice received 1 mg of CMP (CM group) or GMP (GM group) per gram of body weight together with 0.3 μg/g of CT. The effect of the adjuvant *per se* was evaluated using two control groups, one receiving CT in phosphate buffer saline (PBS) (CT group) and another group receiving only PBS (C group). Six weeks after the first feeding mice were fasted overnight and challenged intragastrically. The C, CT and CM groups received two doses of CMP (30 mg/mouse) given 30 minutes apart. Similarly the GM group received two doses of GMP (30 mg/mouse) given 30 minutes apart. Previous experiments performed by our group had shown that control groups (C, CT) could be challenged either with CM or GM with identical results (data not shown).

Thirty minutes after the last challenge, mice were killed by intraperitoneal administration of sodium pentothal (50 mg/kg), and blood was collected by cardiac puncture in ethylenediaminetetraacetic acid containing tubes. Spleen and intestine were removed from each mouse and weighed.

Evaluation of Symptoms

Diarrhea was used as the gastrointestinal symptom of interest. Diarrhea usually occurred within 7 days of the second dose. Animals suffering from diarrhea in at least two consecutive weeks were considered positive cases.

Immediately after challenge, hypersensitivity responses were evaluated by using a scoring system modified slightly from previous reports as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth and pilar erecti; 3 = decreased activity with increased respiratory rate; 4 = wheezing and labored respiration; 5 = cyanosis around the mouth and the tail; 6 = death (30).

Evaluation of symptoms was performed by two independent investigators who were not directly involved in the study and who were unaware of the animals' experimental group.

Measurement of CM or GM-Specific IgG1 in Plasma

Blood was centrifuged at 3500 rpm for 10 minutes at 4°C and plasma aliquots were collected and frozen at −80°C. Levels of CM or GM-specific IgG1 were measured as previously described (30). Briefly, 96-well plates were coated with 20 μg/mL of protein from cow or goat milk in coating buffer (Na_2CO_3 0.5 M). After overnight incubation at 4°C, plates were washed three times with wash solution (50 mM Tris, 0.14M NaCl, 0.05% Tween 20) and blocked with blocking solution (50 mM Tris, 0.14 NaCl, 1% BSA). Then, plasma samples (1:10 dilutions) were added to the plates and incubated for 1 hour at room temperature. Plates were then washed three times and 100 μL of goat anti-mouse IgG1 antibody conjugated with peroxidase (Bethyl Laboratories) was added for an additional 1 h at room temperature. Staining was performed with TMB (Sigma Chemical) for 30 minutes at room temperature and in the dark, stopped with 0.1N H_2SO_4 and plates were read at 450 nm. Results were expressed as optical density (OD) ± SD. All analyses were performed in duplicate.

Determination of Plasma Histamine Levels

Plasma histamine levels were determined by an enzyme immunoassay kit (IBL Laboratories, Hamburg, Germany) following manufacturer recommendations.

Spleen Cells Culture

Spleens were removed from all mice 30 minutes after the last challenge and homogenized in complete culture medium (DMEM plus 10% fetal bovine serum [FBS], 1% penicillin/streptomycin). After centrifugation at 1500 rpm for 5 minutes erythrocytes were lysed with a lysing solution (NH_4Cl, 1.7 M; $KHCO_3$, 0.12 M; EDTA, 0.009 M) for 15 minutes at 4°C. Resting cells were counted by using a hemacytometer and cultured to perform proliferation and stimulation assays in current culture medium (DMEM + 10%FBS). Cells were incubated at 37°C in a humidified 5% $CO_{2\ atm}$.

Proliferation Assay

Spleen derived lymphocytes were cultured in 24-well plates (2×10^6 cells/well) in 1 mL of medium. Lymphocytes from every mouse were divided into three wells, one incubated in the absence of proteins (basal), one in the presence of CMP (100 μg/mL) and one in the presence of GMP (100 μg/mL). [^{3}H]-tymidine (1 μCi/mL) (Amersham Biosciences, Arlington Heights, IL) was added to each well. After 48 h plates were centrifuged at 1500 rpm for 5 minutes and supernatants were discarded. To fix cells 500 μl of ice-cold 70% methanol was added to each well. After three washes in ice-cold 10% trichloroacetic acid, cells were solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation using a 2100 Tri-Carb Packard (Meriden, CT) scintillation counter. Each point was performed in duplicate and the results were expressed as the mean ± SD.

Analysis of Cytokine Production

Spleen derived lymphocytes were cultured in six-well plates (10×10^6 cells/well) in 5 mL of medium and incubated during 24 h in the presence or absence of ConA (5 μg/mL), LPS (50 μg/mL), CMP or GMP (100 μg/mL). The supernatants were collected after 48 hours of incubation. Levels of IgA, IgG1, interleukin (IL)-2, IL-4, tumor necrosis factor-α and interferon (IFN)-γ were determined by ELISA according to the manufacturer instructions (Bethyl and Biosource).

Analysis of Gene Expression by Reverse Transcription-Polymerase Chain Reaction

Total mRNA was isolated from cultured spleen cells by using Trizol Reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. The reversed transcription was performed by using the Reverse Transcriptase AMV (Roche, Indianapolis, IN) as described elsewhere (31). First-strand cDNAs were either stored at −20°C or used for the polymerase chain reaction (PCR) step. PCR reactions (25 μL total volume) were performed as previously reported (31). Once the PCR reactions were completed, 20 μL of each reaction mixture was used for electrophoresis on 2% agarose gels, and DNA bands were visualized with ethidium bromide staining and ultraviolet irradiation. Murine oligonucleotides primers used in the PCR were purchased from MWG-Biotech AG (Ebersberg, Germany) and the nucleotide sequences were as follows: IFN-γ, forward 5′-TGGAGGAACTGGCAAAAGGATGGT-3′, reverse 5′-TTGGCACAATCTCTTCAC-3′; IL-4, forward 5′-ACGAGGTCACAGGAGAAGGGA-3′, reverse 5′-GGAGCAGCTTATCGATGAATC-3′; β-actin, forward 5′-TGGAATCCTGTGGCATCC-3′, reverse 5′-AACGCAGCTCAGTAACAGTCC-3′.

Statistical Analysis

Statistical significance ($P < 0.05$) was calculated by Student's *t*-test in case of parametric parameters such as the results of ELISAs and proliferation assays. All tests were performed with one tail following the two-sample equal variance model (homoscedastic). For nonparametric parameters (score, intestine weight/body weight ratio and number of mice with diarrhea) Mann-Whitney *U*-test or Fisher's exact test were used to determine statistical significance ($P < 0.05$). Finally, the Spearman test was used for the statistical analysis of the correlation between antigen-specific IgG1, serum histamine concentration and hypersensitivity score.

RESULTS

The percent of animals with diarrhea was higher in CM-sensitized than GM-sensitized mice ($P = 0.02$ versus CM) (Table 1). Diarrhea in neither group was so severe as to cause a significant decrease in body weight or in food intake (data not shown). We did not expect more severe symptoms because this is a model of milk atopy rather than a model of milk allergy.

After 6 weeks of sensitization, mice were challenged intragastrically with two doses of 30 mg/mouse of CM (C, CT and CM groups) or GM (GM group) without cholera toxin. Hypersensitivity symptoms became evident within 15 to 30 minutes. The severity of the reaction was scored as indicated above. As with diarrhea, the most severe allergic reactions were also observed in CM-sensitized mice (Fig. 1). Mice sensitized with GM showed weaker reactions and control mice showed no symptoms of hypersensitivity after CM challenge. Mice of the CT-group had a mild reaction that was probably attributable to weak allergic responses to allergens in food as a consequence of the immunostimulation caused by cholera toxin. The average severity score of the reaction in the CM group was 2.923 ± 1.115 (mean ± SD), which was significantly higher ($P < 0.01$) than that observed in the CT control group (0.615 ± 0.506). In con-

TABLE 1. *Number of mice suffering from diarrhea*

Group	No. of mice	%
C	0/13	0[a]
CT	3/13	23.1[a]
CM	8/13	61.5[b]
GM	1/13	7.7[a]

Groups with different letters significantly differ from each other.
C = control mice; CT = cholera toxin control mice; CM = cow's milk sensitized mice; GM = goat's milk sensitized mice.

trast, the score of the GM group was 1.153 ± 1.068, which was significantly lower ($P = 0.01$) than that of the CM group but did not statistically differ from that of the CT control group ($P = 0.206$) (Fig. 1). Taken together, these results indicate that we achieved a sensitization that was clearly less severe in the GM group.

Thirty minutes after the second dose of challenge, mice were killed under anesthesia and the intestine of each mouse was removed and weighed. The ratio of intestine weight to body weight was significantly higher in the CM group (0.113 ± 0.008) compared with the GM group (0.100 ± 0.009) ($P = 0.02$) and the CT group (0.98 ± 0.004) ($P = 0.01$) although no edema nor ulceration was grossly observed. This might indicate a weak intestinal inflammation caused by CM sensitization consistent with the gastrointestinal symptoms of the CM group.

Because lymphocytes play a key role in the development of CMPA, the spleen of each mouse was removed and weighed. Spleen-derived lymphocytes were counted and cultured to perform proliferation assays and to study their response to different stimuli. Lymphocytes from CM-sensitized mice significantly increased their proliferation ratio when CMP was added to the culture medium (Fig. 2). In contrast, GMP failed to induce a significant increase in the proliferation ratio of lymphocytes from the GM group. However, this increase in the proliferation ratio in the CM group was not reflected in spleen weight and number of cells, which were similar in all groups (data not shown), suggesting that the number

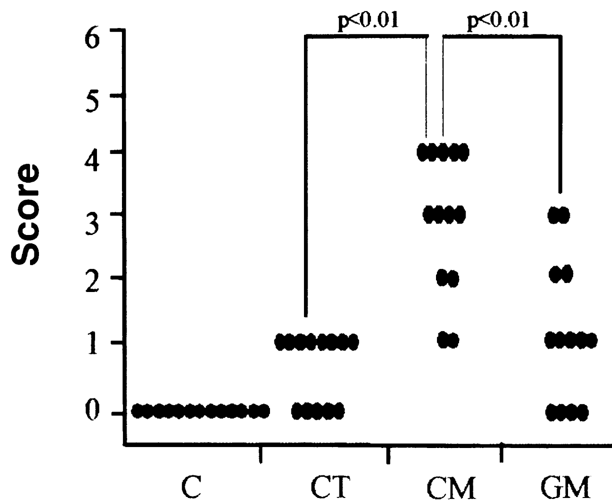

FIG. 1. Score of the hypersensitivity symptoms. Mice (n = 13 per group) were challenged intragastrically with cow milk (C, CT and CM groups) or goat's milk (GM group). Thirty to 40 minutes later, the symptoms of hypersensitivity were scored blinded on a scale from 0 (no symptoms) to 6 (death) as described in the text. Black circles indicate individual mice from one independent and significant experiment. Mann-Whitney *U*-test was performed to determine statistical significance ($P < 0.05$). CM = cow milk sensitized mice; GM = goat's milk sensitized mice; C = control mice; CT = cholera toxin control mice.

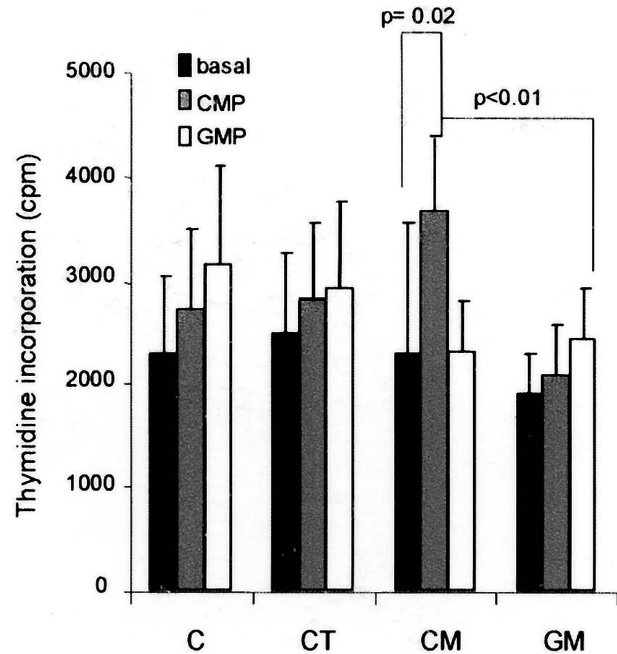

FIG. 2. Lymphocyte proliferation assay. Spleen was removed from each mouse (n = 13 per group) immediately after killing, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured in the presence or absence of cow milk proteins (CMP) or goat's milk proteins (GMP) at 100 µg/mL. [^{3}H]-thymidine (1 µCi/mL) was added before incubation during 48 h. Proliferation ratios were determined as [^{3}H]-thymidine incorporation as described in the text. Results are expressed as thymidine incorporation (cpm) ± SD. Student's *t*-test was used to determine statistical significance ($P < 0.05$). CM = cow milk sensitized mice; GM = goat's milk sensitized mice; C = control mice; CT = cholera toxin control mice.

of lymphocytes sensitized to CMP represents a low percentage of total spleen cells.

The production of cytokines by spleen cells derived from CM-sensitized and GM-sensitized mice was analyzed to investigate what type of lymphocyte response was responsible for the development of milk atopy. Spleen-derived lymphocytes were cultured in the presence or absence of a nonspecific stimulus (ConA). After 48 hours in culture, IL-4 levels were significantly increased in lymphocytes from CM-sensitized mice, compared with those from control mice and GM-sensitized mice ($P < 0.05$) (Fig. 3*A*). Furthermore, IFN-γ production by spleen cells from CM-sensitized mice was significantly lower than those from GM-sensitized mice ($P < 0.05$) (Fig. 3*C*).

Consistent with these results, the analysis of cytokine expression by reverse transcription-PCR showed an increased expression of IL-4 (Fig. 3*B*) and a decreased expression of IFN-γ in spleen cells from CM-sensitized mice (Fig. 3*D*). In all cases GM-sensitized mice showed a response similar to that of the control groups.

To study systemic effects of sensitization, plasma was collected from each mouse immediately after killing.

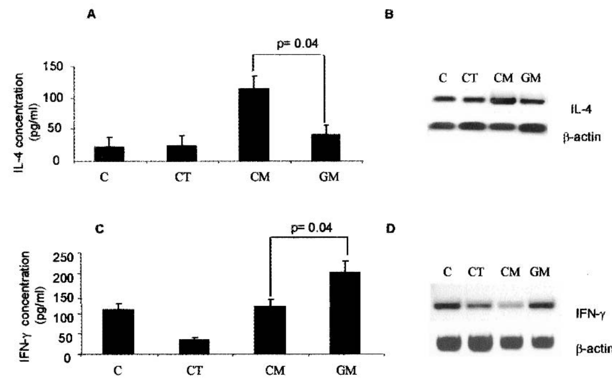

FIG. 3. Cytokine production by T-cells. Spleen was removed from each mouse (n = 13 per group) immediately after killing, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured with or without Con A (5 μg/mL) as a T-cell stimulus. After 48 h in culture, supernatants were collected and interleukin-4 (**A**) or interferon-γ (**C**) concentration was measured by an ELISA method. Results are expressed as concentration (pg/mL) ± SD. Student's *t*-test was used to determine statistical significance ($P < 0.05$). Total lymphocytes mRNA was also obtained and expression of interleukin-4 (**B**) and interferon-γ (**D**) was analyzed by reverse transcription-polymerase chain reaction. β-actin was used as a control of gene expression. CM = cow milk sensitized mice; GM = goat's milk sensitized mice; C = control mice; CT = cholera toxin control mice.

Plasma levels of antigen-specific IgG1 were measured by ELISA. As shown in Figure 4*A*, CM induced a higher synthesis of CMP-specific IgG1 ($P < 0.05$) compared with mice from the CT and C control groups. In contrast, GM did not induce such an IgG1 response, as GMP-specific IgG1 levels were similar to those of the CT-treated and control mice. Antigen-specific IgE levels in the same samples were too low to be detected by the ELISA method used (data not shown). Moreover, no differences were observed in total plasma amounts of IgG, IgG1 or IgE in all groups (data not shown).

Plasma histamine levels were significantly increased in CM-sensitized mice when compared with CT treated mice (Fig. 4*B*). However, the plasma histamine concentration in GM-sensitized mice was similar to that of the control groups. We observed that mice with a higher score had also the highest levels of histamine liberation ($P < 0.01$, r = 0.520, n = 49) (Fig. 5), thus suggesting that the higher histamine liberation was responsible for the symptoms of allergy.

DISCUSSION

To our knowledge this is the first animal study comparing cow milk and goat milk as first protein sources after breast-feeding. Although no ideal model exists, we used a previously described animal model (30) that exhibits the characteristics of type 1 hypersensitivity reactions to compare the allergenicity of cow milk and goat milk. The purpose of using this model was to mimic human food allergy by provoking food hypersensitivity by oral ingestion. Other animal models have not been reported to provoke systemic reactions (32,33) or have been induced by parenteral challenge (34), so they did not adequately mimic human food allergy.

To bypass the tendency of mice to develop oral tolerance, we used CT, which has been reported to stimulate a Th2 response and the production of IgG1 antibodies (35,36). Furthermore, Balb/C mice were used because this strain of mice is prone to develop Th2 responses easier than other strains (37).

In the animal model used in this study IgG1 seems to be responsible for mast degranulation and histamine release. This is in agreement with previous studies dem-

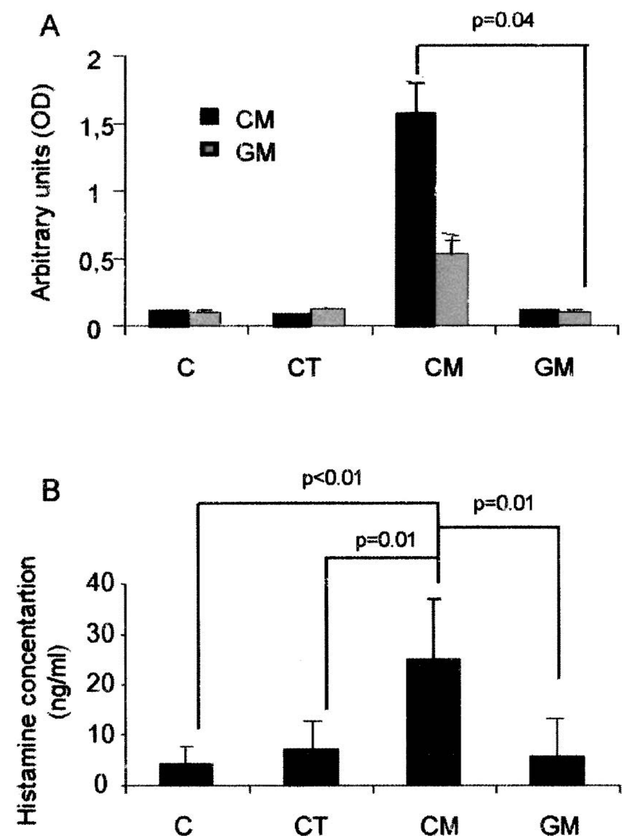

FIG. 4. A, serum levels of antigen-specific immunoglobulin G1 differ between CM-sensitized mice and the other groups. Sera from all mice (n = 13 per group) were collected 30 minutes after the last challenge. CM-specific and GM-specific immunoglobulin G1 were determined by ELISA. Results are expressed as OD ± SD. Student's *t*-test was used to determine statistical significance ($P < 0.05$). B, serum histamine levels differ between CM-sensitized mice and the other groups. Sera from all mice (n = 13 per group) were collected 30 minutes after challenge. Histamine concentration was determined by ELISA. Results are expressed as concentration (ng/mL) ± SD. Student's *t*-test was used to determine statistical significance ($P < 0.05$). CM = cow milk sensitized mice; GM = goat's milk sensitized mice; C = control mice; CT = cholera toxin control mice.

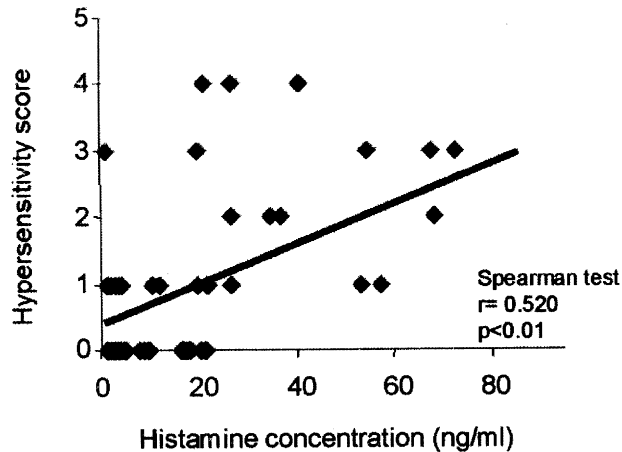

FIG. 5. Correlation between serum histamine concentration and hypersensitivity score. Spearman test was used to determine statistical significance ($P < 0.05$) and the correlation coefficient (r) between serum levels of histamine and hypersensitivity score 30 minutes after the last challenge. Black circles indicate individual mice (n = 49).

onstrating that IgG1 can mediate type I hypersensitivity in mice (38,39). It is well known that mouse IgG1 binds to Fc-receptors in mast cells and promotes degranulation when reacting against a specific antigen, thus increasing histamine levels. This increase in histamine liberation is probably responsible for the symptoms observed in this study. Whereas CM provoked a strong IgG1 response after intragastric challenge, GM failed to induce such a response. Thus histamine release was higher in CM-sensitized mice and was probably responsible for hypersensitivity symptoms.

T cells and cytokines play an important role in regulating allergic diseases such as asthma (40,41). However, the extent of T cells involvement in food allergy is unclear. In our study, CM stimulation of spleen cells from mice allergic to CM induced an increase in the proliferation ratio of spleen cells. It also induced a significant increase in the synthesis of IL-4 but a decrease in the production of IFN-γ, both crucial cytokines for Th2 and Th1 responses, respectively. These findings suggest that development of CMPA might be mediated by a Th2 response, as previously reported (30). In CM-sensitized mice the increase in IL-4 production might stimulate IgG1 synthesis by specific B cells, thus explaining the higher levels of CMP-specific IgG1 levels in CM-sensitized mice. In contrast, GM did not induce a similar response, thus suggesting that GM-sensitized mice developed a less severe hypersensitivity reaction.

In conclusion, we compared CM and GM as first sources of protein after weaning in a murine model of food allergy. Our results suggest that GM is less immunogenic than CM because GM induced a weaker Th2 response. Further studies are needed to determine whether GM could be a suitable substitute for CM in infant formulas.

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