



Allergic responses induced by goat milk α_{S1} -casein in a murine model of gastrointestinal atopy

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ABSTRACT

Up to 3% of young children develop milk allergy and this may influence the development of immune-mediated diseases in later life. One protein that has been associated with allergic reactions to ruminant milk is α_{S1} -casein (CN). Studies suggest that goat milk with low levels of α_{S1} -CN may reduce allergenicity of milk, but the dose response to α_{S1} -CN has not been confirmed. In this study, we examined the immune response to varying levels of goat α_{S1} -CN in a mouse model of gastrointestinal allergy. BALB/c mice (aged 5 wk) were given intraperitoneal injections with α_{S1} -CN and aluminum as adjuvant at 1 and 3 wk to sensitize mice to the antigen. In wk 5, groups of fasting mice ($n = 8/\text{group}$) were challenged 4 times on alternate days by intragastric gavage with saline or 2, 10, or 20 mg of α_{S1} -CN. Serum levels of specific IgE, IgG₁, and IgG_{2a} antibodies and mouse mast cell protease-I were determined. Interleukin-4, IL-10, and IFN- γ responses to 48-h activation with antigen were measured in cultured splenocytes. We determined that mice sensitized with α_{S1} -CN had higher titers of specific IgG₁ and IgE antibodies compared with controls; however, groups challenged with differing doses of α_{S1} -CN did not differ. The group challenged with the highest dose of α_{S1} -CN had a 10-fold increase in mouse mast cell protease-I compared with the group challenged with saline. Both IL-4 and IL-10 were produced in a dose-dependent manner by cultured splenocytes incubated with α_{S1} -CN. Overall, α_{S1} -CN stimulated the production of cytokines associated with allergic disease in a dose-dependent manner. Thus, milk with lower levels of α_{S1} -CN should contribute to a lesser antigenic burden.

Key words: milk protein, α_{S1} -casein, goat, allergy

INTRODUCTION

Allergic diseases have increased significantly over the last 20 to 30 yr and represent a growing health problem in both Western and developing countries (Chamlin et al., 2009; Anandan et al., 2010; Simonsen et al., 2011). During infancy, 5% of infants exhibit food hypersensitivity in the first year of life (Garcia-Careaga and Kerner, 2005). Food allergy during infancy is a well-recognized major risk factor for development of other allergy-related diseases such as asthma, persistent atopic dermatitis, and hypersensitivity to various environmental allergens (Kulig et al., 1998; Halcken, 2004). Therefore, prevention or reduction of food allergy occurrence would effectively lessen the development of other allergy-related diseases.

Allergy to cow milk in infants and young children affects 2 to 3% of children (Høst, 2002). Although goat milk is similar to cow milk, evidence suggests that it is less allergenic than cow milk, although the precise incidence of goat milk allergy is unknown. A study investigating the cross-reactivity of goat milk in children allergic to cow milk found that 24 of the 26 children tested reacted to goat milk but that the amount of milk required to induce a reaction was 5 times greater than the amount of cow milk needed (Bellioni-Businco et al., 1999). Reports also exist of people who are allergic to goat or sheep milk but tolerant of cow milk (Umpiérrez et al., 1999; Ah-Leung et al., 2006). Combined, these data suggest that allergic reactivity to goat milk differs from that of cow milk.

One factor that may contribute to the differences in allergenicity of goat and cow milk is the different profile of caseins. Cow and goat milks both contain the 4 main casein classes: α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN, but the level of α_{S1} -CN in goat milk may range from high (7 g/L), to medium (3.2 g/L), low (1.2 g/L), or absent, depending on the polymorphism of the gene (Grosclaude and Martin, 1997). Cows only have high levels (~12 g/L) of this casein (Farrell et al., 2004), except for animals with the rather common G variant associated with lower expression of the specific CN (Caroli et al., 2009).

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Various studies have reported that goat milk with lower α_{S1} -CN levels has reduced allergenic effects. Bevilacqua et al. (2001) used guinea pigs to demonstrate a reduced allergic response with goat milk containing low α_{S1} -CN (0.7 g/L) compared with high α_{S1} -CN (7 g/L). Lara-Villoslada et al. (2004) performed studies in BALB/c mice also using milk with low levels of α_{S1} -CN (1.4 g/L) and showed reduced allergenicity compared with cow milk containing normal levels (7 g/L). Using sera from children allergic to cow milk, Ballabio et al. (2011) conducted in vitro and skin prick testing to compare the reactivity with milk from goats with varying α_{S1} -CN genotypes and showed a lesser reaction to low α_{S1} -CN goat milk.

To build on the evidence that milks with low α_{S1} -CN may be beneficial for reducing milk allergy, we investigated the dose response to goat α_{S1} -CN in a mouse model of gastrointestinal allergy. Mice were first sensitized to the protein by intraperitoneal injection and then challenged orally with increasing doses of goat α_{S1} -CN. The immune response was assessed by several humoral and cellular parameters.

MATERIALS AND METHODS

Purification of α_{S1} -Casein from Goat Milk

Isolation of α_{S1} -CN from goat milk was achieved by ion exchange chromatography using previously published methods (Jaubert and Martin, 1992; Gómez-Ruiz et al., 2004) with some modifications. Pooled milk, from individual goats previously determined to produce milk with high α_{S1} -CN, was obtained from a local dairy goat farm. Milk was heated to 45°C and centrifuged at $1,650 \times g$ for 30 min and then the fat was removed. Skim milk was reheated to 45°C, the pH was adjusted to 4.2 with 2 M HCl, and the milk was stirred for 30 min and then centrifuged at $1,650 \times g$ for 15 min to recover the casein precipitate. The casein was washed (4 times) by resuspending in purified water (10 times the original milk volume) and stirring for 1 h before recentrifugation at $1,650 \times g$ for 15 min. The casein was then freeze-dried, vacuum packed, and stored at 4°C until required. The casein was dissolved at 11.5 g/L in 25 mM sodium formate buffer containing 7.5 M urea and 0.8 mM DL-dithiothreitol (DTT), pH 4.0, stirred for 2 h at room temperature (21°C), and then filtered through a 1.0- μ m filter. A total of 28 g of casein was applied to an Amicon column (90 \times 12.5 cm) containing 800 mL of SP Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) equilibrated with 75 mM sodium formate, 7.5 M urea, and 64 μ M DTT, pH 4.0. Elution was carried out at room temperature (21°C) at a flow rate of 24 mL/min by a stepwise gradient of 0.11

M, 0.16 M, and 1.0 M NaCl in 75 mM sodium formate, 7.5 M urea, and 64 μ M DTT, pH 4.0, using 4.0, 4.0, and 2.5 column volumes, respectively. The protein peaks were collected in separate fractions and ultra-filtered using a 10-kDa membrane to remove urea and sodium chloride before freeze-drying. The fractions were evaluated by SDS-PAGE.

Characterization of Column Fractions by Gel Electrophoresis

Column fractions were assessed by SDS-PAGE, based on published methods (Laemmli, 1970), with some modifications. Samples were prepared in Laemmli's sample buffer containing β -mercaptoethanol (5%) and boiled for 5 min. The SDS-PAGE was performed using a 10 to 20% separating gel (Criterion, Bio-Rad Laboratories, Auckland, New Zealand). Samples were run on the gel for 60 min at a constant voltage of 150 V, and then stained by using the colloidal Coomassie Blue G 250 method (Neuhoff et al., 1988). Casein subclasses were identified by molecular weight based on literature sources (Jaubert and Martin, 1992) and purity was assessed by the number of bands; that is, a protein with high purity would run as a single protein band.

Mice and Experimental Protocol

Animal experiments were performed in accordance with the guidelines of the New Zealand National Animal Ethics Advisory Committee for the use of animals in research, testing, and teaching. BALB/c mice were housed in specific-pathogen-free conditions and maintained on a dairy-free diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Indianapolis, IN) and water ad libitum. Female mice 5 to 6 wk old were allocated to 5 groups ($n = 8$). In wk 1 and 3 of the trial, groups received intraperitoneal injections containing 0 (control) or 50 μ g of goat α_{S1} -CN (prepared as described above) and 1.5 mg of aluminum (Imject Alum, Pierce Biotechnology, Rockford, IL). In wk 5, the mice were fasted overnight before oral challenge once daily on 4 alternate days with 0, 2, 10, or 20 mg of goat α_{S1} -CN administered by gavage in 250 μ L of saline solution. Control mice also received 4 oral gavages with saline only. Mice were monitored for 2 h following the oral gavage to assess hypersensitivity responses, which were scored using a modified reported system (Gill et al., 2001): 1 = smooth coat, bright-eyed and alert, no symptoms; 2 = active, bright-eyed, slightly hunched and jumpy, scratching and rubbing around nose and head; 3 = mouse not as alert or active, hunched when sitting and eyes closed or squinting, fur slightly ruffled; 4 = hunched and still, little interest in the environment,

fur noticeably ruffled; 5 = nonreactive to stimuli, fur bottle-brush appearance, mouse cold to touch. Mice were also observed for signs of diarrhea.

On the final day, mice were euthanized by CO₂ asphyxiation and cervical dislocation 2 h following the oral gavage. Heparinized blood was obtained by cardiac puncture, centrifuged for 10 min at $1,650 \times g$ and room temperature (21°C). Plasma was stored at -20°C until analyzed for specific and total IgG₁, IgG_{2a}, and IgE, and mouse mast cell protease-I (MMCP-I). The spleens were excised and cells isolated for culture (see below).

Mouse Mast Cell Protease-1 and Total Antibody Measurements

Levels of MMCP-1 in mouse plasma were measured by ELISA (MS-RM3 ELISA kit, Moredun Scientific Ltd., Midlothian, UK) according to the manufacturer's recommended protocol. Total IgG₁, IgG_{2a}, and IgE ELISA kits were purchased from Bethyl Laboratories (Montgomery, TX) and used according to the manufacturer's recommended protocol.

Specific Antibody Measurement

Specific antibody titers were measured using a non-competitive indirect ELISA protocol based on previously described methods (Clark and Engvall, 1980; Hodgkinson et al., 1995). All washes were carried out by using an automated plate washer (Dynex AM60 MRW, Dynex Technologies, Chantilly, VA) using PBS containing 0.05% (vol/vol) Tween 20 (PBS-T), all samples and reagents were diluted in PBS-T containing 1% (wt/vol) BSA (A-7906, Sigma-Aldrich, Auckland, New Zealand). All incubations were at room temperature (21°C), unless otherwise stated. Microtiter plates (Maxisorb F-96, Nalge Nunc International, Roskilde, Denmark) were incubated overnight at 4°C with 10 $\mu\text{g/mL}$ goat α_{S1} -CN (prepared as described above) in bicarbonate buffer (50 mM NaHCO₃, pH 9.8). After washing, plates were incubated with PBS-T containing 1% (wt/vol) BSA. Serial dilutions of test samples (1:100; 1:1,000; 1:10,000; 1:100,000; 100 μL) were then incubated for 1 h and then washed 3 times. Positive (plasma pooled from mice gavaged with α_{S1} -CN) and negative (untreated-mouse plasma) controls were run with each plate. Goat anti-mouse IgE, IgG₁, or IgG_{2a} conjugated with horseradish peroxidase (Bethyl Laboratories) were added to plates at 1:10,000; 1:200,000, and 1:5,000 dilutions, respectively, and incubated for 1 h. After washing, plates were incubated with substrate solution (TMB One Component HRP Microwell substrate, BioFX Laboratories, Owings Mills, MD) for 20 min and the reaction stopped with 2 M H₂SO₄. Opti-

cal density (OD) was measured at 450 nm using an automated plate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Antibody titers for test samples were defined as the reciprocal of test sample dilution that produced an OD equal to 50% of the maximum OD above background of the positive control. Results were expressed as titer units of antibody.

Splenocyte Culture and Cytokine Measurement

A cellular suspension was produced by mincing individual spleens between 2 sterile glass slides. The red blood cells were lysed with ACK Lysing Solution (Bossuyt et al., 1997) and the splenocytes extensively washed and resuspended in RPMI 1640 containing 10% fetal calf serum, 0.1% penicillin, 0.1% streptomycin, and 0.1% glutamine. Cells (5×10^6 cells/mL) were cocultured in 24-well plates at 37°C in 5% CO₂, and stimulated with goat α_{S1} -CN (100 $\mu\text{g/mL}$). Concanavalin A (1 $\mu\text{g/mL}$) and LPS (2 $\mu\text{g/mL}$) were used for positive controls and unstimulated cells for background controls. Supernatants were harvested at 48 h and assayed for IL-4, IL-10, and IFN- γ concentrations by ELISA (OptEIA mouse ELISA set, BD, Auckland, New Zealand), according to the manufacturer's recommendations. Detection limits were 7.8, 31.3, and 31.3 pg/mL for IL-4, IL-10, and IFN- γ , respectively.

Statistical Analysis

Data are presented as means \pm SEM. The Student *t*-test was performed for comparison between 2 groups. When comparing 2 groups with unequal variance, data were transformed to logs before *t*-test analysis. *P*-values < 0.05 were considered statistically significant.

RESULTS

Purity of α_{S1} -Casein

Stepwise elution of casein from the ion-exchange column with increasing concentrations of salt produced 4 protein peaks. The second peak eluted with 0.16 M NaCl contained α_{S1} -CN. Assessment of the α_{S1} -CN fraction by SDS-PAGE showed the protein to be a single band with an estimated purity of >98% (Figure 1). Identification of the protein was confirmed by molecular weight size on SDS-PAGE (Figure 1) and chromatographic profiling by HPLC (data not shown).

Oral Allergen-Induced Response

Oral challenge with goat α_{S1} -CN in mice presensitized to the protein induced increasing severity of physical

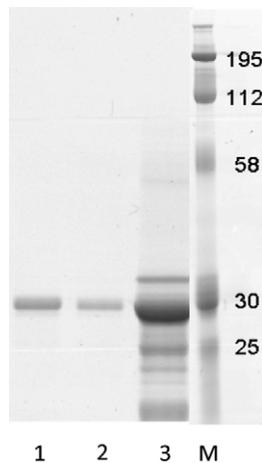


Figure 1. Sodium dodecyl sulfate-PAGE characterization of purified α_{S1} -CN. To assess the purity and confirm the identity of the α_{S1} -CN, 1.0 μ g (lane 1) and 0.5 μ g (lane 2) of the purified protein were run on an SDS-PAGE gel with a goat milk sample (lane 3). Molecular weight markers were run alongside (lane M).

symptoms with increasing number of gavages (data not shown). The severity of symptoms was generally low with scores ranging from 1 to 2, although one animal in the group receiving 10 mg of α_{S1} -CN scored 3 to 4 with the final gavage. At no time was diarrhea induced in any of the mice, even at the fourth gavage with the highest antigen dose (20 mg of α_{S1} -CN). Control mice displayed no physical reactions.

Only High Oral Doses of α_{S1} -Casein Induce a Mouse Mast Cell Response

Overall, levels of MMCP-1 were low in plasma samples. Multiple oral gavages with 20 mg of α_{S1} -CN in mice presensitized to α_{S1} -CN produced a 20-fold increase in serum MMCP-I compared with control (nonsensitized) mice gavaged with saline, and a 10-fold increase compared with presensitized mice gavaged with saline (Figure 2).

Antibody Response to α_{S1} -Casein

Mice presensitized with α_{S1} -CN had higher total IgE and IgG_{2a} concentrations in serum compared with control mice, whereas total IgG₁ was unchanged (Figure 3). Multiple oral challenges with allergen did not further increase total antibody levels. For IgE- and IgG₁-specific α_{S1} -CN antibodies, titer values were higher in mice presensitized to allergen compared with control mice, with the greatest increase in IgG₁ titers (Figure 4). Overall, a small trend was observed for titers to increase with increasing oral dose. Specific IgG_{2a} α_{S1} -CN titers were not significantly increased in presensitized

mice compared with the control mice except for the group orally dosed with 20 mg of protein.

Induction of Cytokine Secretion in Splenocytes by α_{S1} -Casein

Culture supernatants from cells isolated from the individual mouse spleens were analyzed for IFN- γ , IL-4, and IL-10. Cytokine responses to mitogen stimulation (concanavalin A and LPS) in splenocytes from control mice were similar to responses from mice presensitized with allergen (data not shown). Restimulation with allergen in vitro induced no cytokine response in splenocytes from control mice (Figure 5). In contrast, when splenocytes from presensitized, orally challenged mice were restimulated with α_{S1} -CN, levels of IFN- γ , IL-4, and IL-10 were all increased compared with that of control mice (Figure 5). For IL-4 and IL-10, levels increased in a dose-dependent manner; mice orally challenged with higher doses of α_{S1} -CN produced higher levels of cytokine in their splenocytes when restimulated with allergen. The same dose response was not apparent for IFN- γ .

DISCUSSION

The mouse model of gastrointestinal atopy used in this trial was first developed using ovalbumin (OVA) as a model antigen similar to other studies (Brandt

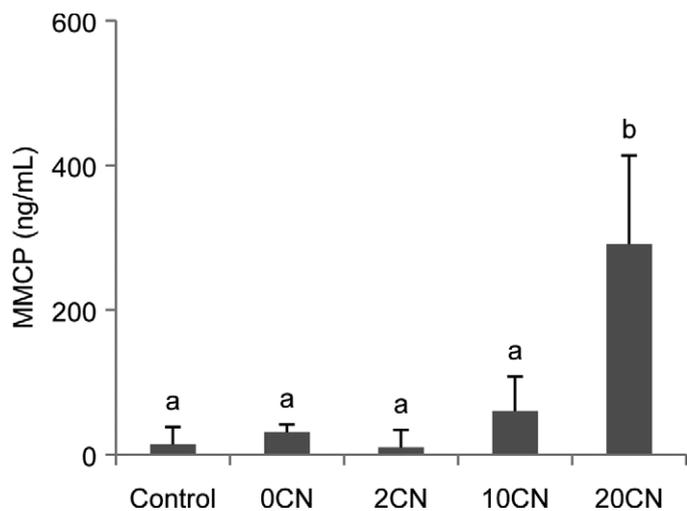


Figure 2. Plasma levels of mouse mast cell protease-I (MMCP-I). Mice presensitized intraperitoneally with 50 μ g of α_{S1} -CN were challenged 4 times with saline (0CN), 2 mg (2CN), 10 mg (10CN), or 20 mg (20CN) of α_{S1} -CN. Nonsensitized control mice were challenged with saline. Plasma was collected from all mice ($n = 8$ per group) 2 h after the last challenge, and MMCP-I was measured by ELISA. Results are represented as group mean \pm SEM. Different lowercase letters indicate statistically significant differences ($P < 0.05$) determined by Student t -test.

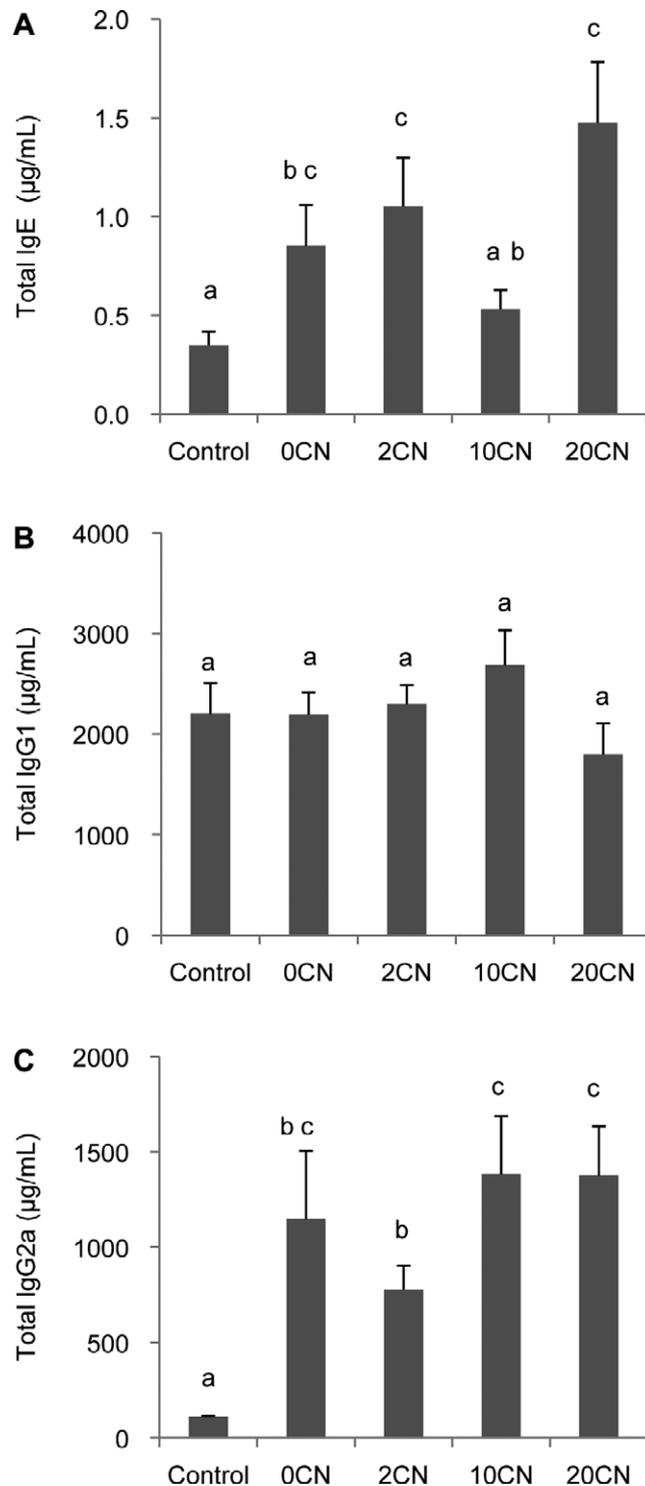


Figure 3. Plasma levels of total IgE (A), IgG₁ (B), and IgG_{2a} (C). Mice presensitized intraperitoneally with 50 μg of α_{S1} -CN were challenged 4 times with saline (0CN), 2 mg (2CN), 10 mg (10CN), or 20 mg (20CN) of α_{S1} -CN. Nonsensitized control mice were challenged with saline. Plasma was collected from all mice ($n = 8$ per group) 2 h after the last challenge, and total antibodies were measured by ELISA. Results are represented as group mean \pm SEM. Different lowercase letters indicate statistically significant differences ($P < 0.05$) determined by Student *t*-test.

et al., 2003). Mice presensitized with OVA/alum and subsequently exposed to repeated doses of intragastric OVA exhibited a dose-dependent allergic response illustrated by gastrointestinal anaphylaxis with symptoms of diarrhea usually occurring within 30 min of exposure (Brandt et al., 2003). However, using α_{S1} -CN as allergen, we found that the physical response of presensitized mice to oral challenge was weak, with few obvious symptoms and no diarrhea apparent. One reason for this may have been the maximal protein dose that the mice received. In trials in which OVA was used as antigen, a dose-dependent allergic response was observed with an optimal dose of 50 mg of OVA (Brandt et al., 2003). In this trial, however, the highest dose of α_{S1} -CN that we could achieve was 20 mg due to low solubility of the protein.

Another reason for the weak physical response to the α_{S1} -CN observed after oral challenge in presensitized mice was the influence of allergen-specific IgG antibody on IgE-mediated anaphylaxis. Intestinal inflammation due to allergenic foods is accompanied by T cell helper-type 2-associated antibodies and cellular responses that have been shown to be largely dependent on IgE and mast cells (Forbes et al., 2008). One study found that when IgG antibody concentration was high (induced by either active immunization or passive administration) and the challenge allergen dose was low, the IgG-specific antibody inhibited IgE-mediated anaphylaxis by allergen interception (Strait et al., 2006). In the present study, the magnitude of the increase in IgG₁-specific α_{S1} -CN titers was greater than that of the IgE-specific α_{S1} -CN titers.

Interestingly, Knight et al. (2007), using this same mouse model, demonstrated that oral challenge with a low dose of allergen, although not inducing physical symptoms, did prime the gut. After repeated oral low-dose challenge, a subsequent single challenge with high dose OVA induced diarrhea in 40 and 100% of mice primed with 1 and 5 mg of OVA, respectively. Knight et al. (2007) also showed that the cytokine responses to allergen in mesenteric lymph nodes preceded gut responses and suggested that T cells activated in the mesenteric lymph nodes mediated the gastrointestinal response.

In this trial, although the response to antigen was reduced as measured by some parameters, an immune cell priming effect of the oral dose of α_{S1} -CN was evident in the spleen cells. Spleen-derived cells from groups of mice challenged to increasing oral dose of antigen responded in a dose-dependent manner to α_{S1} -CN re-exposure in culture with cytokines associated with Th₂ cells. This suggests that α_{S1} -CN oral dosing had generated memory T helper 2 cells, similar to that induced by OVA in the mesenteric lymph nodes (Knight et al.,

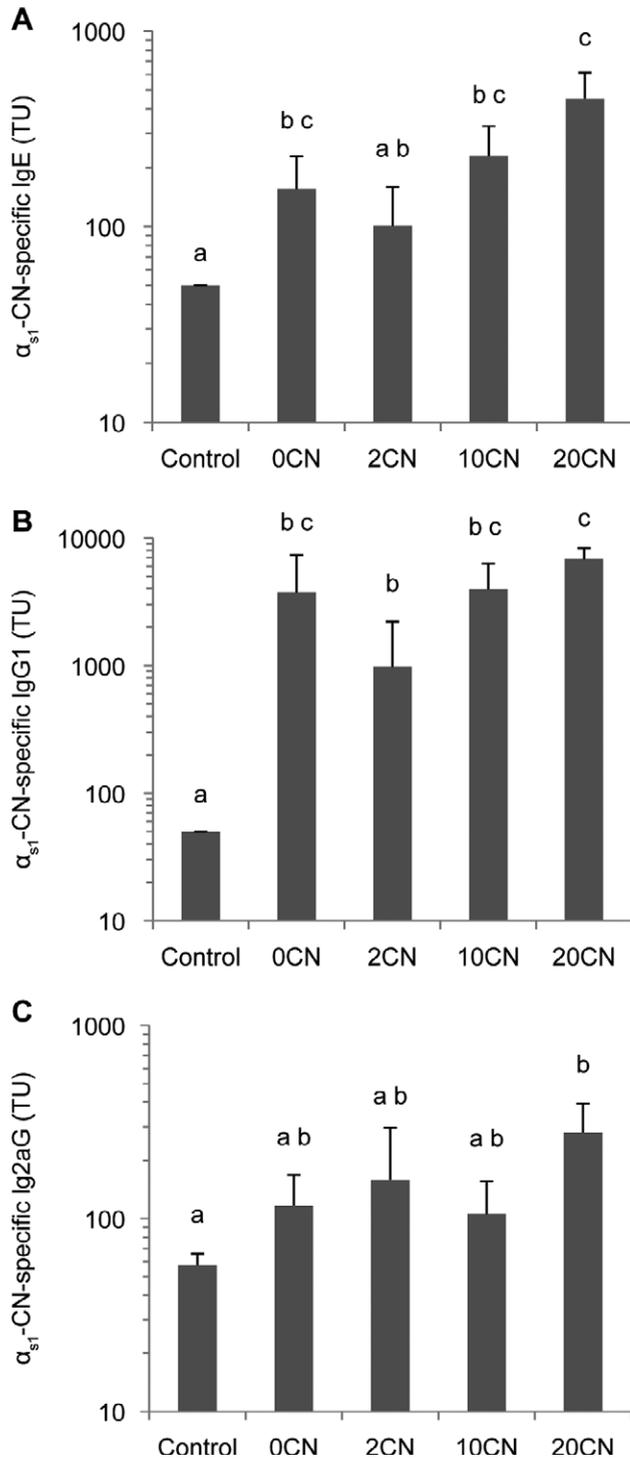


Figure 4. Plasma levels of α_{S1} -CN-specific IgE (A), IgG₁ (B), and IgG_{2a} (C). Mice presensitized intraperitoneally with 50 μ g of α_{S1} -CN were challenged 4 times with saline (0CN), 2 mg (2CN), 10 mg (10CN), or 20 mg (20CN) of α_{S1} -CN. Nonsensitized control mice were challenged with saline. Plasma was collected from all mice ($n = 8$ per group) 2 h after the last challenge, and α_{S1} -CN-specific antibodies were measured by ELISA. Results are expressed as arbitrary titer units (TU) and represented as group mean \pm SEM. Different lowercase letters indicate statistically significant differences ($P < 0.05$) determined by Student *t*-test.

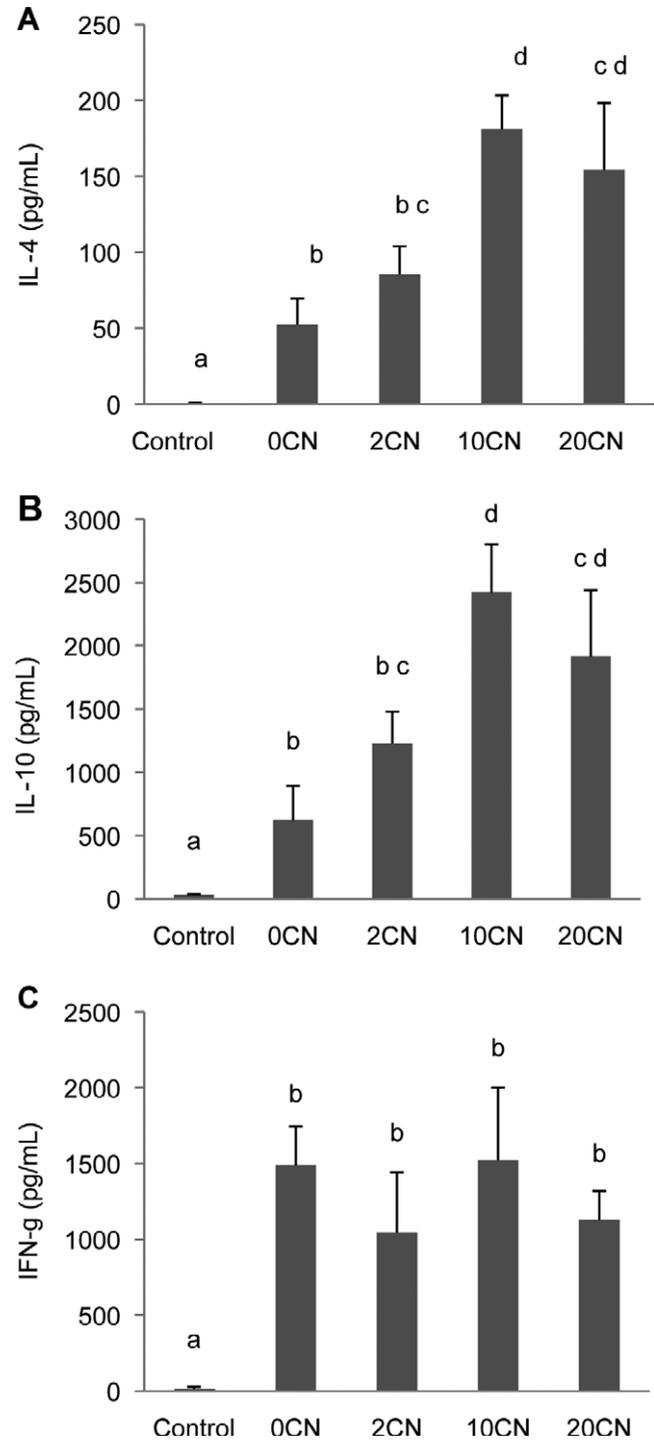


Figure 5. Culture supernatants concentrations of IL-4 (A), IL-10 (B), and IFN- γ (C). Mice presensitized intraperitoneally with 50 μ g of α_{S1} -CN were challenged 4 times with saline (0CN), 2 mg (2CN), 10 mg (10CN), or 20 mg (20CN) of α_{S1} -CN. Nonsensitized control mice were challenged with saline. Splens were collected from each mouse ($n = 8$ per group) immediately after killing, 2 h after the last challenge. Isolated spleen cells from individual mice were cultured in the presence of 100 μ g/mL α_{S1} -CN for 48 h. Cytokines concentration in supernatants was measured by ELISA. Results are represented as group mean \pm SEM. Different lowercase letters indicate statistically significant differences ($P < 0.05$) determined by Student *t*-test.

2007). Based on these data, we conclude that α_{S1} -CN had the potential to induce an allergic response in the gastrointestinal tract in this model.

To our knowledge, this is the first study that has directly investigated the allergenicity of α_{S1} -CN purified from goat milk. Other studies have shown that milk from goats that produce lower levels of α_{S1} -CN is potentially less allergenic than milk from goats with higher levels of this protein (Bevilacqua et al., 2001; Lara-Villoslada et al., 2004; Ballabio et al., 2011). We confirm that reduced levels of α_{S1} -casein in milk would be beneficial for the overall allergenicity of milk. The levels in goat milk can vary up to 10-fold, which is the difference between the lowest and highest dose used in the present study.

Goat milk with low levels of α_{S1} -CN has other characteristics that may also contribute to the modulation of the allergic response. Bevilacqua et al. (2001) showed a reduced sensitization to β -LG in guinea pigs fed a diet incorporating goat milk with low α_{S1} -CN (0.7 g/L) compared with a diet incorporating goat milk with high α_{S1} -CN (7 g/L), even though levels of β -LG were higher in the low α_{S1} -CN milk, suggesting that other factors may be involved. Bevilacqua et al. (2001) proposed that biochemical interactions between caseins and β -LG are different in low α_{S1} -CN milk and may improve the digestion of β -LG. Furthermore, studies have shown that α_{S1} -CN has a role in the secretion process of milk proteins (Chanat et al., 1999; Le Parc et al., 2010), thereby influencing the composition of milk proteins and milk lipids (Neveu et al., 2002; Devold et al., 2010). The apocrine pathway of secretion for goat milk compared with the merocrine secretion process described for cow milk has been attributed to the α_{S1} -CN polymorphism (Neveu et al., 2002). This pathway of milk secretion produces cell fragments or cytoplasmic particles in goat milk similar to those observed in human milk (Linzell and Peaker, 1971; Malatesta et al., 2000; Boutinaud and Jammes, 2002). Further studies that compare the minor milk components of high or low α_{S1} -CN goat milk would be useful in understanding the complex mechanisms underlying the different immune responses to milk antigens in the gastrointestinal tract.

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