Small Ruminant Research 112 (2013) 191–198

Contents lists available at SciVerse ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Comparative evaluation of allergic sensitization to milk proteins of cow, buffalo and goat

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ARTICLE INFO

Article history:
Received 25 May 2012
Received in revised form 8 November 2012
Accepted 9 November 2012
Available online 4 December 2012

Keywords:
Allergic sensitization
Hypersensitivity
Milk proteins
Humoral response
Lymphocyte proliferation index
Cow
Buffalo and goat

ABSTRACT

Milk hypersensitivity due to adverse reactions to milk proteins is among the major public concern of food allergies. In present investigation, comparative assessment of humoral and cell mediated immune responses in mice associated with allergenicity of major milk proteins (casein and β-lactoglobulin) of cow, buffalo and goat were carried out. Caseins of all three species of dairy animals appeared to be less immunogenic and allergenic than their corresponding β-lactoglobulins. Cow milk proteins (caseins and β-lactoglobulin) were observed to increase protein specific IgE sensitization as well as lymphocyte proliferation index significantly ($P < 0.01$) as compared to buffalo and goat milk proteins. On the other hand, buffalo and goat milk proteins similar to cow's proteins raised remarkably higher ($P < 0.01$) immunogenic response in terms of IgG levels than the negative control group. The results obtained were correlated with B and T cell epitopes of milk proteins cited in literature after multiple alignment *in silico*. Thus present study suggests cow milk proteins (β-lactoglobulin and casein) are highly allergenic among the milk proteins of three species of dairy animals.

1. Introduction

Milk is considered nature's most perfect food as it contains almost all the substances essential for human nutrition. Consumption of dairy products is also associated with beneficial health effects beyond its pure nutritional value (Silanikove et al., 2009, 2010). However, cow's milk is also among the most common causes of food allergy in the early years of life. Approximately 1–2% of the adult population and up to 8% of children below the age of three (Helm and Burks, 2000) suffers from cow's milk hypersensitivity. It is an adverse immunological response to cow's milk proteins affecting the cutaneous, intestinal or respiratory systems. Clinical symptoms involve immediate or delayed reactions either due to IgE dependent or cell mediated type IV hypersensitivity. Early infancy is a critical period for the development of immunological memory, wherein the T helper (Th) balance converts from the Th2-skewed immunity to Th1 cell-type responses under the influence of genetic and environmental factors. Studies suggest that postnatal maturation of the immune system in atopic individuals is attenuated, and atopic infants show an imbalance in Th1/Th2 immune responses by excessive IgE responses (Holt and Jones, 2000). A number of regulatory T-cells appear to be involved in regulating Th responses, and defects in regulatory T-cell responses have been linked to allergic diseases (Stock et al., 2006; Taylor et al., 2006; Wing and Sakaguchi, 2006). The action of allergens is based on their recognition by IgE (or possibly IgG) through linear or conformational epitopes (B cell epitopes). So far, no general structural characteristic of allergens have been discovered: linear epitopes differ in the amino acid number and sequence and an exchange of a single amino acid may lead to disappearance of IgE binding, and further there is no general conformational pattern discernible. Since there

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http://dx.doi.org/10.1016/j.smallrumres.2012.11.028

are no medical treatments currently available for curing food allergies, the best way to prevent unintended exposure to a food allergen is the complete avoidance of the offending food. For various reasons such avoidance may not always be possible, and in certain instances, impossible. Although the gross composition of cow's, buffalo's and goat's milk is very similar, slight differences in protein fractions result in changes in immunological and physico-chemical properties. Human milk proteins have many significant differences in their amino acid composition compared to the milk proteins of animals. Children with a high-risk of atopy or diagnosed cow milk allergy sometimes receive food substitutes. This can include other milk source proteins such as formulae containing goat milk with altered proportion of casein content and/or soy milk, cow milk hydrolysates with destroyed allergenic epitopes or amino-acid based formulae (Walker-Smith, 2003; El-Agamy, 2007; Silanikove et al., 2010) to reduce allergic reactions. It is therefore interesting to consider the adequacy of those alternatives by comparing their biophysical and allergenic properties to cow milk. Similarly the true prevalence of cow and goat milk allergy is not exactly known and is surrounded by controversy. Moreover, no relevant scientific data regarding allergenic sensitization with buffalo milk is available. So the present study has been carried out to compare the allergenic sensitization to major milk proteins (caseins and β-lactoglobulin) of cow, buffalo and goat.

2. Materials and methods

2.1. Mice

Three to four-week-old male Swiss albino mice were maintained in the Small Animal House of the National Dairy Research Institute (N.D.R.I.), India. The animals were housed in plastic polypropylene cages and kept at room temperature in a sterilized condition and were placed on a special milk-free diet. All procedures were approved by the Institutional Animal Ethical Committee.

2.2. Milk sample collection

Whole cow milk was procured from Experimental Dairy Plant (EDP), National Dairy Research Institute, Karnal. Buffalo and goat milks were procured from cattle yard, N.D.R.I., Karnal.

2.3. Isolation of milk proteins

2.3.1. Total caseins (CSN)

Separation of CSN was carried out by isoelectric precipitation method. In brief, collected cow, buffalo and goat milk were centrifuged at 5000 × *g* for 20 min at 4 °C, to obtain skim milk. Immediately after collection, skim milk was warmed up to 40 °C, pH was adjusted to 4.6 in case of cow and buffalo milk and 4.1 for goat milk respectively with 1 N HCl followed by stirring for 30 min. The resultant precipitate was separated by filtration through four layers of cheese cloth and washed three to four times with distilled water, until the pH came to 7.0.

2.3.2. β-Lactoglobulin (β-LG)

Separation of β-LG was carried out by the method described earlier Alomirah and Alli (2004). Briefly, whey solution collected after the removal of precipitated CSN was clarified from fine CSN particles by centrifugation at 5000 × *g* for 15 min at 4 °C. The whey proteins except β-LG were precipitated by the addition of 7% NaCl at pH 2.0 after 20 min of incubation at room temperature. β-LG remained in supernatant after the removal of precipitated whey proteins by centrifugation at 10,000 × *g*, 15 min, at 4 °C. The precipitation process was repeated thrice to remove all traces of other whey proteins from β-LG solution. Sugar and salts were removed from this preparation by dialysis using 8 kDa cutoff cellulose ester membrane at 4 °C for 24 h. β-LG preparation was concentrated by lyophilization at −40 °C for 48 h.

2.4. SDS-PAGE of fractionated milk proteins for purity analysis

The concentration of proteins in various samples was estimated by the method of Lowry et al. (1951). The purity of fractionated milk proteins (CSN and β-LG) were checked by SDS-PAGE using 15% resolving gel of polyacrylamide by the method of (Laemmli, 1970) loading equal concentration (20 μg/well) of fractionated milk protein in each well).

2.5. Experiment design and sample collection

Six groups of six mice each were sensitized thrice at weekly intervals by intraperitoneal (i.p.) injection of 20 μg/animal of whole CSN or β-LG obtained from cow, buffalo and goat milk using 2% of $Al(OH_3)$ as adjuvant in PBS. Negative control group mice were only given adjuvant in PBS while positive control mice were sensitized with same dose of ovalbumin. One week after the last injection, mice were sacrificed and blood was obtained by cardiac puncture and sera were separated by centrifugation at 1500 × *g* for 10 min. Sera collected were stored at −20 °C until further analysis. Spleen was collected after opening the peritoneal cavity and transferred to 15 ml of sterile centrifuge tube containing 2 ml of RPMI-1640 media. It was washed and made free from connective tissue and adipose tissue.

2.6. Measurement of total IgE and IgG levels

Total IgE and IgG in serum were determined by using ELISA kit (Koma Biotech, Seoul, Japan). Operating procedures were strictly followed according to the manufacturer's instructions. In brief, total IgE and total IgG levels from serum were determined using quantitative sandwich ELISAs. To detect these antibodies, plates were coated with 1 μg ml^{-1} of goat anti-mouse IgE or IgG, respectively. Serial dilutions of serum were added and then followed by the addition of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE or IgG. Plates were allowed to develop with the TMB substrate (3,3′,5,5′-tetramethyl diamine benzidine containing 0.03% H_2O_2) and reaction was finally stopped with 100 μl of 2 M H_2SO_4. Plates were read at 450 nm against 630 nm as reference wavelength.

2.7. Measurement of milk protein-specific serum IgE titers

Whole CSN and β-LG specific IgE antibodies were detected using enzyme-linked immunosorbent assay (ELISA). Plastic microtiter plates (Nunc, Copenhagen, Denmark) were coated with 100 μg/ml of protein (CSN or β-LG) in PBS by overnight incubation at 4 °C. The plates were blocked by incubation for a further 1 h at 37 °C with 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS (for plates containing CSN) or 1% fish gelatin (for the plates containing WP). Mouse serum samples diluted two times serially in PBS were added to wells and incubated for 1 h at 37 °C followed by washing with PBS containing 0.05% Tween 20 and further incubation for 1 h at 37 °C with peroxidase-labeled goat anti-mouse IgE diluted 1:10,000 in PBS. Enzyme substrate TMB was added to each well and the reaction stopped after 15 min by the addition of 100 μl of 2 M H_2SO_4. Substrate conversion was measured as optical density at 450 nm using an automated ELISA reader. Antibody titer was determined by dilution (log 2) till absorbance reached 0.150 O.D value.

2.8. Lymphocyte proliferation index (LPI)

Aseptically collected spleen tissues were gently teased with sterile needles and forceps to release splenocytes into the RPMI-1640 media. Tissue suspensions were allowed to stand for 2 min to sediment large tissue clumps. The upper portion containing splenocytes was collected and centrifuged at 1000 × *g* for 5 min at 4 °C. Suspension was incubated with erythrocyte lysis buffer (0.17 M Tris–HCl and 0.16 M NH_4Cl, pH 7.2) for 1 min and washed twice by centrifuging as above with RPMI-1640 medium. Cell viability was checked by trypan blue (0.4% solution). Viable splenocytes (10^7 cells/ml) were finally cultured in heat inactivated 10% fetal calf serum enriched RPMI-1640 medium supplemented with mitogens (LPS (50 μg ml^{-1}) or Con A (25 μg ml^{-1}) or 50 μg ml^{-1} isolated milk proteins (β-LG or CSN) of cow, buffalo and goat, respectively and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Then, 10 μl