

## ORIGINAL ARTICLES

### ANTIGENICITY OF COW'S MILK PROTEINS IN TWO ANIMAL MODELS

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#### ABSTRACT

Antigenicity of proteins found in cow's milk is age dependent. This is primarily due to infants possessing a more permeable intestinal wall than that in adults. Thus infants may acquire cow's milk allergy during their first year of life. While milk antigen specific IgE may cause allergy in susceptible subjects, there is some evidence indicating that milk antigen specific IgG may play some role in chronic disease development. The purpose of this study was to determine the antigenicity of cow's milk proteins in two animal models and to recommend the more sensitive one, as an evaluation tool, to assess the antigenicity of a potential hypoallergenic formula. A crude extract of cow's milk was injected either to young male rabbits or BALB/C mice in four doses. Pure standard proteins of cow's milk were also injected to separate groups of animals to use their anti sera in later stages. The polyclonal pooled serum was then used to evaluate the antigenicity of the extract by indirect enzyme-linked immunosorbent assay (ELISA), and Western blotting. Both the rabbit and BALB/C murine model demonstrated strong ELISA titres against casein and BSA proteins. However, the rabbit model also had a high antibody response against beta-lactoglobulin ( $\beta$ -lg). The lowest antibody

response was found against alpha-lactalbumin ( $\alpha$ -la) in both animal models and no response against immunoglobulins (Igs) in either model. In Western blotting, rabbit antiserum showed four bands ( $\alpha$ -la,  $\beta$ -lg, caseins and BSA) compared to two bands (caseins and BSA) for mouse antiserum. Considering the allergenicity of these proteins in genetically prone subjects, it may be wise to exclude food sources of caseins as well as major whey proteins (BSA), from the diet of infants with a family history of atopy during the first year of life. The rabbit hyperimmunization model was more sensitive than the murine model in detecting antibodies against milk proteins. Thus, the rabbit model should be employed when evaluating the antigenicity of a hydrolysate formula.

**Keywords:** Antigenicity, Cow's milk proteins, Animal model

### INTRODUCTION

Milk is the first food of mammalian infants. The rapid development of hygienic milk technology, dating originally from the end of the nineteenth century, has ensured the widespread use of clean, relatively safe, often pasteurized, cow's milk<sup>(1)</sup>. However, despite all precautions, some infants did not thrive on cow's milk-based food but developed severe gastrointestinal symptoms and even collapse. It was later realized that the foreign proteins in cow's milk were responsible for the symptoms. Still, the high nutritional value of cow's milk proteins and their convenient availability have secured their position as the major protein source for infant foods in Western developed countries. The allergenicity of cow's milk proteins has remained a major concern in infant nutrition<sup>(2)</sup>. There is some evidence indicating that cow's milk hypersensitivity manifestations are not confined just to those resulting from IgE-mediated reactions<sup>(3)</sup>.

Once the diagnosis of cow's milk allergy is established, every one agrees that it must be treated by eliminating the offending food but there is less agreement over what is to be fed in its place<sup>(4)</sup>. Considering the risk of inadequate bone mineralization because of long-term suppression of dairy products intake<sup>(5)</sup> one of the hypoallergenic formulas may be prescribed to the cow's milk allergic infant. A proposed definition of a

hypoallergenic formula for cow's milk-allergic children is that it does not provoke any allergic signs or symptoms in at least 90% of infants with documented cow's milk protein allergy when tested in double-blind, placebo-controlled food challenge trials<sup>(4)</sup>. There are several food-processing approaches to modify allergenic potential of milk-based formulas<sup>(6)</sup>. However, before these formulas undergo clinical testing for allergenicity, non-clinical testing can characterize their molecular and physical properties to assess antigenicity<sup>(7)</sup>. The objective of this study was to compare antigenicity of cow's milk proteins in two animal models and to introduce the more sensitive one to evaluate candidate hypoallergenic formula.

### MATERIALS AND METHODS

Briefly, a cow's milk extract was made in phosphate-buffered saline (PBS). Rabbits and BALB/C mice were then immunized with the extract. The resulting polyclonal sera were then used in an indirect enzyme immunoassay and Western immunoblotting.

#### Preparation of cow's milk extract

Three milliliters of 2.5% fat pasteurized cow's milk was diluted with 30ml 0.15M phosphate-buffered saline (PBS) pH 7.2 in sterile centrifuge tubes. The tubes were then incubated on rotator for

about 16hr (overnight) at 4°C. After overnight incubation, the tubes were centrifuged at 2500g for 10min, the supernatants were recovered and transferred to fresh sterile tubes and centrifuged again at 17000g for 15min at 4°C. Finally, the supernatants were filter-sterilized through 0.45µm and 0.22µm cartridge filters<sup>(8)</sup>. The amount of protein present in the extract was determined by the Bradford protein assay<sup>(9)</sup>.

### Preparation of the anti sera

Young male rabbits (~2.5 months) and BALB/C mice (~3 weeks) were injected intramuscularly (IM) and intraperitoneally (IP), respectively, with either cow's milk extract or standard solutions of caseins (Merck), α-la (Fluka), β-Ig (Fluka) or BSA (Merck) in four separate dosages. Each of the five above-mentioned protein solutions was injected either into two rabbits or 10-12 mice. The first injection was with Freund's complete adjuvant and the others with Freund's incomplete adjuvant (Sigma). The amount of protein injected into rabbits was 1.5mg for the first two injections and 1mg for the last two injections. The amount of protein injected into mice was 200µg, 100µg, 100µg and 50µg in four injections. The time interval between injections was two weeks for the second injection and one week for the others<sup>(10)</sup>. Two days after the last injection, rabbits were bled through cardiac puncture while mice were bled by retro-orbital bleeding. Whole blood was allowed to clot at room temperature for 1 hour before centrifugation at 1300g for 15min to obtain serum. Serum samples from each animal model, within protein injections, were pooled together to give a total of five serum samples for each animal model.

### Enzyme-linked immunosorbent assay (ELISA) for antibody titration

Wells of a 96 well microplate (Nunc) were coated with 100µl of protein solution containing 10µg/ml of cow's milk extract, β-lactoglobulin (β-Ig), or bovine serum albumin (BSA), 5µg/ml of caseins or 20µg/ml of α-lactalbumin (α-la) in PBS. The coating was done at 4°C overnight (~16 hr). After washing with PBS/Tween 20 (PBST), serial dilutions were made from the anti sera prepared in the previous

stage. The plate was then incubated for one hour at room temperature. The plate was washed again with PBST and then 100µl sheep anti-rabbit or anti-mouse Ig conjugated with horseradish peroxidase (HRP) (Sigma) diluted with PSBT to 1:5000 was transferred to the wells. After an hour of incubation period, wells were washed and 100µl of the O-phenylene diamine (OPD) in citrate buffer was then added to each well. Optical density was read using a plate reader (Organon Technica, Reader 210) at 492 nm. All tests were done in duplicate.

### SDS-PAGE and Western blotting

Electrophoresis was performed with 3% stacking gel and 6-20% gradient running gel, 25mA for ~6hr at room temperature. The amount and volume of protein solution transferred to each well were ~5-15µg and 25-50µl, respectively. To compare the protein distribution of whole cow's milk with the extract by electrophoresis, 20µl cow's milk was diluted with 80µl sample buffer and heated in boiling water for 3 min; 10µl was then loaded on to the gel. The gel was then stained with Coomassie blue. This method has been used successfully for human milk electrophoresis<sup>(11)</sup>. Western blotting was done with a current of 45mA for ~16hr at room temperature. Samples blotted on nitrocellulose were incubated with rabbit or mouse anti sera overnight at 4°C with gentle shaking on shaking rotator. At least four times the titer found for each anti serum in the ELISA was used in this stage. Nitrocellulose stripes were then incubated with sheep anti-rabbit or anti-mouse Ig/HRP for 90 minutes on a shaking rotator at room temperature. Bands were developed on the stripes using 3,3'-diaminobenzidine (DAB) substrate (Sigma).

## RESULTS AND DISCUSSION

All proteins found in cow's milk were present in the extract (fig. 1). In ELISA, the highest serum titer was against casein and BSA in both animal models (fig. 2). In Western blotting, rabbit anti serum could detect four proteins i.e.; BSA, caseins, β-Ig and α-la but only BSA and caseins were detected using mouse anti serum (fig. 3 and 4).

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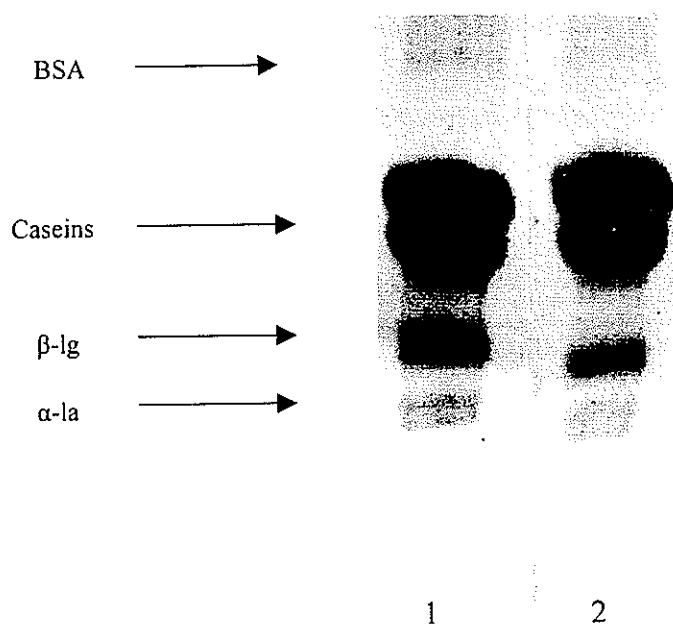


Figure 1. Electrophoretic pattern of cow's milk (lane 1) and cow's milk extract (lane 2). All proteins in the milk are present in the extract with the same proportion.

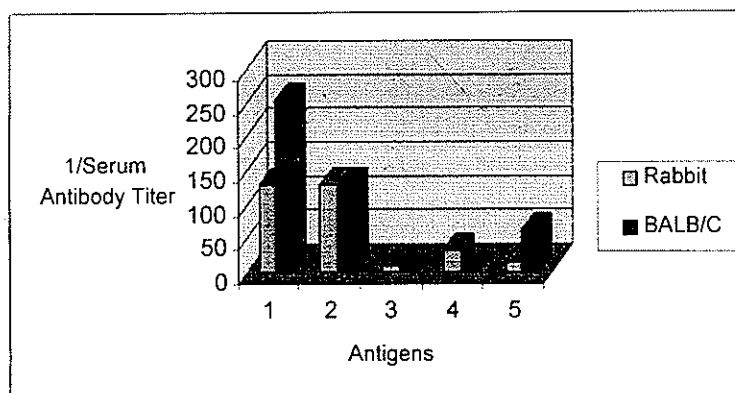
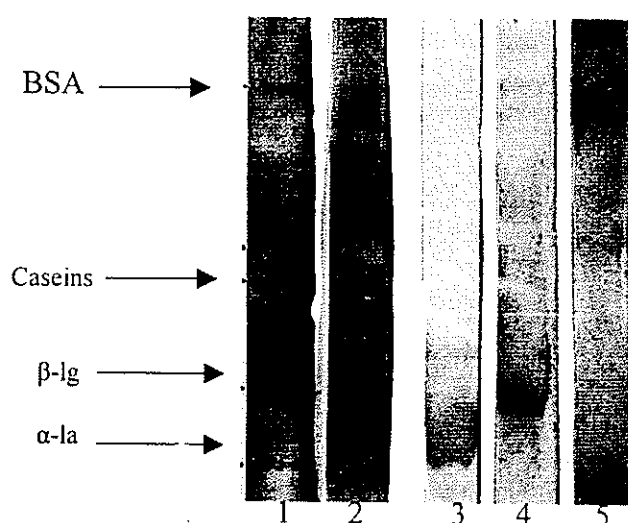
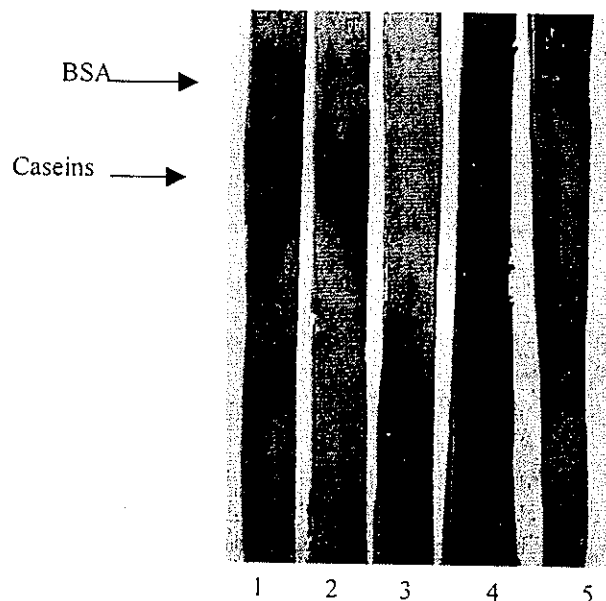


Figure 2. Serum antibody titers against (1) cow's milk extract; (2) caseins; (3) alpha-lactalbumin; (4) beta-lactoglobulin and (5) bovine serum albumin in rabbits and BALB/C mice. The highest serum antibody titers were found against caseins in both animal models.



**Figure 3.** Western blotting with rabbit pooled serum against whole milk crude extract (lane 1) and four pure standard proteins of the cow's milk. As it is seen in the lane 1, rabbit pooled serum could detect four major proteins in cow's milk including bovine serum albumin (BSA), caseins, beta-lactoglobulin ( $\beta$ -Ig) and alpha-lactalbumin ( $\alpha$ -la). Lane 2, caseins; lane 3,  $\alpha$ -la; lane 4,  $\beta$ -Ig (isoforms A and B); lane 5, BSA. See text for details.



**Figure 4.** Western blotting with BALB/C pooled serum against cow's milk whole proteins (lane 1) and purified standard proteins. As it is seen in the first lane, BALB/C pooled serum could detect BSA and caseins out of four major proteins of cow's milk. Lane 2, pure caseins; lane 3, pure beta-lactoglobulin (isoforms A and B); lane 4, pure alpha-lactalbumin; lane 5, pure BSA. See text for details.

Allergenicity of a protein depends on such multiple factors as stability to digestion and the interaction with the intestinal environment. Highly complex interactions exist between antigens, intestinal epithelium and the underlying immune system<sup>(12)</sup>. Indeed the intestinal cells are able to take up and process proteins and possibly to present them directly to the mucosal lymphocytes<sup>(13)</sup>. On the other hand, pathophysiological conditions can modify these interactions<sup>(14)</sup>. Most of the infant formulas used for feeding children not allergic to cow's milk are produced by spray-drying homogenized milk. It was shown that homogenization increases the ability of milk to induce anaphylactic shock in animal models<sup>(15)</sup>.

It would therefore be reasonable to test antigenicity of the hypoallergenic formulas being imported to the Iranian market. There are a large number of methods to assess antigenicity of food proteins based on the various immune responses leading to intestinal or extra-digestive pathologies<sup>(8)</sup>. Western blotting has been used by several investigators to evaluate food antigenicity<sup>(16-18)</sup>. In this study we compared two animal models, rabbit and BALB/C mouse, to assess the antigenicity of cow's milk proteins. The rabbit hyperimmunization model has previously been used to evaluate candidate hypoallergenic protein ingredients. Use of the model has been expanded to include the evaluation of protein hydrolysate formulas<sup>(19)</sup>. In our models, rabbit seemed more sensitive than mouse, as judged by Western blotting. On the other hand, albumin and immunoglobulin (Ig) are the most heat-labile milk antigens though they are not totally destroyed by pasteurization and are among cow's milk major antigens<sup>(20)</sup>. Though BSA has the least amount in cow's milk (only 1% of total protein compared to 9, 3 and 2% for  $\beta$ -Ig,  $\alpha$ -la and Ig, respectively)<sup>(19)</sup>, it showed highest antigenicity, after caseins, in our animal models. Further studies may be needed to assess the antigenicity and allergenicity of this protein in the amount and proportion present in children's food.

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### REFERENCES

1. Harfouche JK, Musaiger AO. Breast-feeding patterns. A review of studies to the Eastern Mediterranean region. WHO, Alexandria p: 13, 1993.
2. Savilahti E, Kuitunen M. Allergenicity of cow milk proteins. *J Pediatr*, 121: S12-S20, 1992.
3. Sampson HA. Adverse reactions to foods. In: Middleton Jr. E, Read C, Ellis E et al., (Eds). *Allergy principles and Practice*. Vol II, Mosby, St. Louis, p: 1661-86, 1993.
4. Walker WA. Adverse food reactions in childhood: summary and future directions. *J Pediatr*, 121: S4-S6, 1992.
5. Infante D, Tormo R. Risk of inadequate bone mineralization in diseases involving long-term suppression of dairy products. *J Pediatr Gastroenterol Nutr*, 30: 310-13, 2000.
6. Lee YH. Food-processing approaches to altering allergenicity potential of milk-based formula. *J Pediatr*, 121: S47-S50, 1992.
7. Leary HL. Non-clinical testing of formulas containing hydrolyzed milk Protein. *J Pediatr*, 121: S42-S46, 1992.
8. Bernhisel-Broadbent J, Yolken RH, Sampson HA. Allergenicity of orally administered immunoglobulin preparations in food-allergic children. *Pediatrics*, 87: 208-14, 1991.
9. Bradford MNA. A Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72: 248-54, 1976.
10. Dresser DW. Immunization of experimental animals. In: Weir DM, Herzenberg LA, Blackwell C, et al. (Eds). *Handbook of Experimental Immunology*. 1. Immunochemistry. 4th ed., Blackwell Scientific Publications, Oxford, p: 8.1-21, 1986.
11. Kunz C, Lönnerdal B. Human-milk proteins: Analysis of casein and casein subunits by anion-exchange chromatography, Gel electrophoresis, and specific staining methods. *Am J Clin Nutr*, 51: 37-46, 1990.
12. Heyman M. Evolution of the impact of food technology on the allergenicity of cow's milk proteins. *Proc Nutr Soc*, 58: 587-92, 1999.
13. Husby S. Normal immune responses to ingested foods. *J Pediatr Gastroenterol Nutr*, 30: S13-S19, 2000.
14. Mayer L. Mucosal immunity and gastrointestinal antigen processing. *J Pediatr Gastroenterol Nutr*, 30: S4-S12, 2000.
15. Poulsen OM, Hau J, Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analyzed by a murine anaphylactic shock model. *Clinical Allergy*, 17: 449-58, 1987.
16. Awazuhara H, Kawai H, Baba M et al. Antigenicity of the proteins in soy lecithin and soy oil in soybean allergy. *Clin Exp Allergy*, 28: 1559-64, 1998.
17. Restani P, Plebani A, Velona T et al. Use of immunoblotting and monoclonal antibodies to evaluate the residual antigenic activity of milk protein hydrolyzed formulas. *Clin Exp Allergy*, 26: 1182-87, 1996.
18. Restani P, Velona T, Plebani A et al. Evaluation by SDS-PAGE and immunoblotting of residual antigenicity in hydrolyzed protein formulas. *Clin Exp Allergy*, 25: 651-58, 1995.
19. Cordle CT, Duska-McEwen G, James LM et al. Evaluation of the immunogenicity of protein hydrolysate formulas using laboratory animal hyperimmunization. *Pediatr Allergy Immunol*, 5: 14-19, 1994.
20. Gjesing B, Løwenstein H. Immunochemistry of food antigens. *Ann Allergy*, 53: 602-8, 1984.