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Immune-modulating properties of horse milk administered to mice sensitized to cow milk

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ABSTRACT

The aim of this study was to examine immune adaptive changes, the expression of innate biomarkers and variations in intestinal microbiota composition after horse-milk administration in BALB/c mice, which were sensitized intraperitoneally using cow β-lactoglobulin and α-casein with aluminum adjuvant. We measured serum antibody IgE levels and the expression of *MCP-1*, *IL-4*, and *TNF-α* in duodenal samples. Changes in immune cell populations in peripheral blood were quantified using flow cytometry, and intestinal microbiota composition was assessed using real-time PCR. We found that horse-milk administration decreased serum IgE levels in sensitized mice. The groups that received horse milk showed an increased population of regulatory T cells ($CD4^+Foxp3^+$). Horse-milk administration decreased the mRNA levels of *IL-4* and resulted in higher transcripts of *TLR-4* in all treatment groups; however, the levels of *MCP-1*, *TNF-α*, and *TLR-2* were unaltered. After horse-milk treatment, we observed a positive effect, with increased numbers of intestinal *Bifidobacterium* spp. We observed immune-modulating properties of horse milk, but future studies should focus on testing horse-milk processing, such as fermentation and destroying most allergenic epitopes to continue research under clinical conditions.
Key words: mare milk, equine milk, BALB/c mice, cow milk protein allergy

INTRODUCTION

The increasing incidence of cow milk allergy highlights the need to develop novel functional foods adapted to allergic consumers. An allergy to cow milk proteins results from an adverse immunological reaction to 1 or more of those proteins. Due to the low homology between cow- and horse-milk proteins, horse milk could be a potential candidate for a health-promoting formula with decreased immunoreactivity and higher nutritional value than formulas based on plant materials.

The proportions of subjects with a high sensitivity to the different cow milk proteins have been estimated: 55% α_{S1}-casein, 90% α_{S2}-casein, 45% β-lactoglobulin, and 0% α-lactalbumin [demonstrated by oral provocation tests, radioallergosorbent test (CAP-RAST, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), and skin prick tests; Natale et al., 2004]. The probability of cross-reactivity between cow milk proteins and those of other animal species has been studied, and it has been shown that IgE from the sera of children allergic to cow milk are capable of recognizing defined epitopes of milk proteins from other animals, such as ewe, goat, and buffalo (Restani et al., 2002). These data demonstrate that milk protein polymorphisms can provoke immunogenic reactions of differing severity and cross-reactivity (Restani et al., 2002; El-Agamy, 2007). Previous research efforts have revealed that antibodies (IgG) directed at cow milk proteins do exhibit cross-reactivity with horse-milk proteins (Fotschki et al., 2015a). However, cross-reactivity between equine and bovine milk proteins occurs at a low level (Businco et al., 2000), probably due to differences between the amino acid sequences (Karabus and du Toit, 2012). Additionally, horse milk has biophysical and biochemical properties that place it closer to human milk than cow milk (Nikkhah, 2012). Its similar total protein content and optimal casein/whey protein ratio, which may be an important factor in determining its allergenicity and richness in essential nutrients (Lara-Villoslada et al., 2005; Fiocchi et al., 2010), supports the potential of using horse milk as a substitute for breast milk. This might be particularly important in certain scenarios to fulfill the nutritional requirements of infants with high sensitivity to cow milk proteins. In line with these studies, recent in vitro data have estimated lower uptake rates of proteolytically resistant peptides from horse

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milk with respect to cow milk, suggesting a potential beneficial role for horse milk in the human diet (Fotschki et al., 2015b). Nevertheless, few attempts have been made to evaluate potential allergic responses to horse-milk proteins. Studies have focused only on the tolerance and allergic potential of horse milk in children with a cow milk allergy (Businco et al., 2000; Fiocchi et al., 2010), and these studies have demonstrated good tolerance and low allergic potential. In a clinical investigation, Businco et al. (2000) indicated that horse milk was tolerated by 96% of tested children with cow milk allergies. However, it was tested orally only 2 times. Research has also been conducted with volunteers drinking horse milk over a long period (Foekel et al., 2009); however, in this case, horse milk was studied for multifactorial atopic dermatitis, not strictly a cow milk allergy.

In this study, we demonstrated the effect of horse milk administered constantly over 23 d in a mouse model of cow milk hypersensitivity. Despite our previous research on horse milk, key questions remain unanswered, such as how horse milk influences gut immunity, in particular the production of T cells with immunosuppressive activity ($\mathbf{T}_{\mathbf{reg}}$) that contribute to intestinal tolerance. In recent years, it has been shown that the gut microbiota play an important role in gut immunity development and contribute to the health of the host with additional metabolic capacities that can have positive effects in increasing the threshold of intestinal tolerance. Experimental models have demonstrated that known beneficial components of the gut microbiota, *Bifidobacterium* spp. and *Lactobacillus* spp., significantly contribute to the regulation of inflammatory processes that can occur at the intestinal level (D'Arienzo et al., 2011; Laparra et al., 2012). However, their role in sensitized animal models with cow milk proteins has not been studied until now.

The objective of this study was to evaluate the effects of horse-milk feeding on the markers of gut health (parameters of innate immunity and microbiota composition), as well as potential changes in adaptive immune cell populations in the peripheral blood of mice sensitized against cow milk proteins.

MATERIALS AND METHODS

Samples

Horse milk was collected from 12 Warmblood mares of the Wielkopolski breed (Genactiv, Poznań, Poland). After the horses were milked, aliquots of fresh milk were used in microbial analysis, and the remaining aliquots were immediately chilled to 4°C and kept frozen at −20°C.

Chemical and Microbiological Analysis of Horse Milk

The chemical composition of the horse milk was analyzed using a MilkoScan FT2 Infrared Milk Analyzer (Foss, Hillerød, Denmark). Plate count agar (105463, Merck, Darmstadt, Germany) was used to assess the total count of viable bacteria (aerobically; 30°C/72 h). Selective media were used to count bacterial cells from the main microbial groups. MacConkey agar (212123, Merck) was used to assess the total number of *Enterobacteriaceae* (aerobically; 37°C/24 h). Kanamycin esculin azide agar (105222, Merck) was used to assess the total number of enterococci (aerobically; 37°C/24 h). De Man, Rogosa and Sharpe agar (PS 60; BioCorp, Warsaw, Poland) was used to assess the total number of mesophilic lactic acid bacteria (aerobically; 30°C/72 h). Dichloran Rose-Bengal Chloramphenicol agar (100466, Merck) was used to assess the total number of mold and yeast cells (aerobically; 25°C/72 h). We pipetted 1 mL of milk onto Petri dishes in duplicate. Plate count was assessed according to the equation $L = \Sigma C/(n1 + 0.1n2)d$, where ΣC was the sum of colonies counted on all the plates retained; n1 was the number of dishes retained in the first dilution; n2 was the number of dishes retained in the second dilution; and d was the dilution factor corresponding to the first dilution. In dishes that contained 30 to 300 colonies, the actual number of a dilution on both plates was given. If plates from the lowest dilutions contained fewer than 30 colonies, the actual number was recorded and expressed as colony-forming units per milliliter.

Animals and Experimental Design

After acclimatizing for 2 wk, female BALB/c mice (n = 32) at 6 wk of age, weighing 18 to 22 g, were randomly distributed into 4 groups (n = 8/group). Animals were housed in cages. Water and a diet free of dairy protein were provided ad libitum (25% yellow corn, 13% red sorghum, 13% white beans, 9% green peas, 8% field peas, 6% yellow millet, 5% sorghum white, 4% husked barley, 3% canola, 2% oats without husks, 2% black sunflower, 2% striped sunflower, 2% buckwheat, 2% kardi, 1% vitamin mix AIN-93, 3% mineral mix AIN-93). According to the standard AIN93G rodent diet, the animals received 19.4% protein, 7.0% total fat, and 56.8% carbohydrate. This formulation satisfied the nutritional requirements for growth in mice. We made some modifications to the original formulation to suit our requirements that the diet be free of dairy protein. In the present study, all groups received 16.89% protein, 6.52% fat, and 59.13% carbohydrates (including dietary fiber and sugars). Milk proteins were also avoided in the diets of mice mothers, during suckling of offspring