Food Research International 59 (2014) 8–15

Contents lists available at ScienceDirect

Food Research International

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

Polyamine supplementation in infant formula: Influence on lymphocyte populations and immune system-related gene expression in a Balb/cOlaHsd mouse model

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ARTICLE INFO

Article history:
Received 19 November 2013
Accepted 30 January 2014
Available online 7 February 2014

Keywords:
Breastfeeding
Immune system
Infant formula
Polyamines
Mouse model

ABSTRACT

The aim of this work was to study whether the proportion of polyamine found in human milk, administered with a commercial infant formula, affected the maturation of the immune system in a BALB/cOlaHsd mouse model. Forty-eight pups (14-days old) were randomly assigned to four-day intervention groups: 1) breast-fed (normal lactation); 2) fed infant formula; and 3) two different groups fed with infant formula supplemented with two different amounts of polyamines. The influence of polyamine administration on lymphocyte populations in the blood, spleen, and mesenteric lymph nodes, as well as on the modulation of immune system-related gene expression in the small intestine, was analyzed. The results demonstrated that polyamine supplementation induced an increase in splenic B cells to levels observed during normal lactation when compared with formula without supplementation. The correlation coefficients for the splenic lymphocyte populations increased with polyamine supplementation, with a dose-dependent effect. Our results demonstrate that polyamines influence gene expression profile, mainly *Cd1d1*, *Cd40*, *Hdac5*, *Hdac7*, *Clcf1* and *Tlr4* compared with normal lactation. In general, the gene expression results verified that the expression of genes associated with immune system was similar in the group with high polyamine supplementation to that observed in the group with normal lactation.

1. Introduction

In the early period of life, both humans and rodents are exposed to a wide variety of microorganisms and dietary antigens, which drive the complete maturation of the intestinal and systemic immune system (Brandtzaeg, 2003).

Breast milk contains bioactive substances, such as polyamines, which are known to be important for the development of the neonate's immune system by providing protection against infections, promoting oral tolerance, and controlling inflammatory responses (Newburg & Walker, 2007). Specific polyamines, including spermine (SPM), spermidine (SPD), and putrescine (PUT) have been identified in the breast milk of mammalian species, but their levels in most infant formulas are significantly lower than those in human milk (Buts, De Keyser, De Raedemaeker, Collette, & Sokal, 1995; Pollack, Koldousky, & Nishioka, 1992; Romain, Dandrifosse, Jeusette, & Forget, 1992).

Dietary polyamines are rapidly absorbed in the small intestine (Seiler & Raul, 2007). They are essential for cell proliferation and differentiation (Löser, 2000) and are involved in DNA, RNA, and protein synthesis (Seiler & Raul, 2007). Previous studies have reported the essential role of polyamines in the development of the intestine (Sabater-Molina et al., 2009), the modulation of intestinal microbiota by polyamines (Gómez-Gallego et al., 2012), and positive effects on the developing spleen in mice (Jolois et al., 2002). The previously reported effects of milk polyamines on the maturation of the intestinal and systemic immune system (Pérez-Cano, González-Castro, Castellote, Franch, & Castell, 2010; Steege, Buurman, & Forget, 1997) suggest that supplementation of manufactured infant formulas with polyamines might improve immune functions of human infants in a manner similar to that observed by breastfeeding.

Only a few studies (Jolois et al., 2002; Pérez-Cano et al., 2010; Steege et al., 1997) on the effects of polyamines during lactation in rodents have been reported. These were performed by orally administering a single polyamine and the pups continuing to be fed by their mothers. Thus, they were not well controlled for real polyamine ingestion due

Abbreviations: FACS, fluorescence-activated cell sorting; FITC, Fluorescein isothiocyanate; HDAC, hystone deacetylase; PE, phycoerythrin; PUT, putrescine; SPD, spermidine; SPM, spermine.

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http://dx.doi.org/10.1016/j.foodres.2014.01.066

to the natural polyamine content in mother's milk that was not reported.

The aim of this work was to investigate whether the proportion of polyamine found in human milk, administered in combination with a commercial infant formula in early-weaned pups, affected the maturation of the immune system in a BALB/cOlaHsd mouse model. For this purpose, the current study evaluated the influence of orally administered polyamines in infant formula on lymphocyte populations in the blood, spleen, and mesenteric lymph nodes, as well as the on the modulation of immune system-related gene expression in the small intestine, in a BALB/cOlaHsd mouse model.

2. Material and methods

2.1. Animals and study design

A total of 48 BALB/cOlaHsd mouse pups were used in the study. The pups were born and raised in a semi-barrier facility of the Central Animal Laboratory, University of Turku as a result of breeding adult dams and sirs that were supplied by a reputed commercial vendor of laboratory rodents (Harlan Laboratories®, Horst, Netherlands).

The pups were housed in groups of two, and grouping was decided based on male–female pup availability from the breeding colony. All the mice were maintained in conventional stainless steel cages (370 cm^2; 26.7 × 20.7 × 14 cm), with solid bottoms and Aspen chips as bedding. Cage change was undertaken twice a week. The room temperature was 23 °C (±3 °C), the relative humidity was 45 to 65%, and artificial lightning was used with a 12 h light/dark cycle (lights on at 6:00 am). Prior to entering the breeding program, the adult mice were acclimatized for at least 30 days. Throughout the breeding period, all the adult mice were fed standard mouse chow (SDS, Special Diet Services, Whitham, Essex, UK) ad libitum, and tap water was provided without restrictions in polycarbonate bottles. The entire mouse colony was judged to be healthy on the basis of the absence of clinical signs and the results of microbiological screening routinely performed on the colony in accordance with European recommendations (Nicklas et al., 2002).

The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland (ESLH-2009-04845/Ym-23) and conformed to the regulations and requirements of the European Union concerning the protection of animals used for scientific purposes.

2.2. Polyamine supplementation

Treatment and handling were performed as described previously (Gómez-Gallego et al., 2012). Fourteen-day-old pups were randomly assigned to one of the following four dietary groups according to dietary treatment: normal lactation group (NL, n = 12), unweaned pups, infant formula group (IF, n = 12), weaned pups fed with commercial infant formula, and weaned pups fed on infant formula enriched with low (T1, n = 12) and high (T2, n = 12) concentrations of polyamines.

PUT (D13208, Aldrich, Steinheim, Alemania), SPD (2626, Sigma, Steinheim, Alemania), and SPM (85590, Fluka, Steinheim, Alemania) were orally administered in the following proportions: 3.38%, 35.48%, and 61.14%, respectively. The concentration levels tested in the two polyamine supplementation groups were: treatment I) 2.10 μg/day PUT, 22.05 μg/day SPD, and 38.00 μg/day SPM (T1); treatment II) 8.40 μg/day PUT, 88.20 μg/day SPD, and 152.00 μg/day SPM (T2). The manufactured formula employed was a commercial infant formula targeted for babies during the first six months, fortified with nucleotides, α-lactalbumin, and ω−3 and ω−6 fatty acids supplied by HERO España S.A., (Alcantarilla, Spain) (infant formula composition was described in Gómez-Gallego et al., 2012). The nonenriched formula and the formula with polyamines (100 μl) were made with warm water following the manufacturer's instructions and given to the pups twice daily by oral gavage as previously described. Early-weaned animals were fed a porridge made with the same IF without the enrichment in polyamines being the polyamines only supplied by oral gavage.

2.3. Sample collection

After the four-day diet intervention, the animals were anesthetized with isoflurane, and blood, mesenteric lymph node, spleen, and small intestine samples were obtained. The blood, lymph node, and spleen were conserved in saline buffer (Dulbecco's phosphate-buffered saline, 1% fetal calf serum, and 0.1% sodium azide) until flow cytometer analysis. The whole small intestine was emptied of the contents for microbiota analysis (not reported here), and the tissue was kept at −80 °C using Trizol® reagent (15596-026, Invitrogen, Paisley, UK) according to the manufacturer's instructions for RNA purification.

2.4. Immunofluorescence and flow cytometry

These analyses were performed as described by Alam, Valkonen, Ohls, Törnqvist, and Hänninen (2010), on the same collection day. Single-cell suspensions of spleen cells and mesenteric lymph node cells were acquired by gently pressing the tissues through a metal mesh. Erythrocytes of spleen and blood samples were removed by hypotonic lysis using 0.2% (wt/vol.) NaCl solution.

Anti-mouse B220 (CD45R) allophycocyanin conjugated (FAB1217A, R&D Systems, Minneapolis, USA), CD4 PE-conjugated, and CD8a FITC-conjugated (Immunotools, Friesoythe, Germany) were used to examine the surface expression of markers. Flow cytometry was performed using fluorescence activated cell sorter (FACS) Calibur® flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and Cell Quest (Becton, Dickinson and Company) software. The cells were characterized according to their light and immunofluorescence characteristics. A single-cell suspension of isolated cells was analyzed by FACS for each sample, and the tissue was assayed. To determine the appropriate distribution of the cell populations, the cell suspension was stained with appropriate combinations of monoclonal antibodies. A maximum of 20,000 gated events were recorded, and the proportions of CD4+, CD8+, and B cells were quantified.

2.5. RT-PCR analysis

The whole small intestine was homogenized in Polytron® PT 10-35 GT (Kinematica, Littau/Lucerne, Switzerland).

For purification of total RNA and cleanup, the RNeasy® Mini Kit (Qiagen, Duesseldorf, Germany) was used. The total RNA concentrations and A260/A280, and A260/A230 ratios were determined using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, USA) spectrophotometer to assure the integrity and the purity of the mRNA.

The expression of genes encoding T-cell and B-cell activation, as well as Toll-like receptors (TLRs), in the small intestinal tissues was assessed using a RT2 First Strand Kit (C-03, SABiosciences Corporation, Frederick, USA) according to the manufacturer's instructions. The real-time PCRs were carried out using 96-well PCR arrays designed for the evaluation of mouse T-cell and B-cell proliferation and the differentiation genes (PAMM-053, SABiosciences) and using a Roche LightCycler 480 (Roche Diagnostics Corporation, Indianapolis, USA) as a cycler platform with a fluorescence detection system. The Mouse T-cell and B-cell Activation RT^2 Profiler™ PCR Array profiles the expression of 84 genes representing T-cell and B-cell activation, a key part of adaptive immunity. This array includes genes involved in B-cell activation, as well as genes involved in B-cell proliferation and differentiation. Genes involved in the activation of T cells and their proliferation and differentiation are also represented, along with genes regulating Th1 and Th2 development and T-cell polarization. Genes involved in the activation of macrophages, neutrophils, and natural killer cells are also included. For cDNA synthesis, 5 μg of total RNA was used with the RT2 First Strand Kit (SA Biosciences)