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Effect of a cocoa diet on the small intestine and gut-associated lymphoid tissue composition in an oral sensitization model in rats☆

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Abstract

Previous studies have attributed to the cocoa powder the capacity to attenuate the immune response in a rat oral sensitization model. To gain a better understanding of cocoa-induced mechanisms at small intestinal level, 3-week-old female Lewis rats were fed either a standard diet or a diet containing 10% cocoa for 4 weeks with or without concomitant oral sensitization with ovalbumin (OVA). Thereafter, we evaluated the lymphocyte composition of the Peyer's patches (PPL), small intestine epithelium (IEL) and lamina propria (LPL). Likewise, gene expression of several immune molecules was quantified in the small intestine. Moreover, histological samples were used to evaluate the proportion of goblet cells, IgA+ cells and granzyme+ cells as well. In cocoa-fed animals, we identified a five-time reduction in the percentage of IgA+ cells in intestinal tissue together with a decreased proportion of TLR4+ IEL. Analyzing the lymphocyte composition, almost a double proportion of TCRγδ+ cells and an increase of NK cell percentage in PPL and IEL were found. In addition, a rise in CD25+, CD103+ and CD62L− cell proportions was observed in CD4+ PPL from cocoa-fed animals, along with a decrease in gene expression of CD11b, CD11c and IL-10. These results suggest that changes in PPL and IEL composition and in the gene expression induced by the cocoa diet could be involved, among other mechanisms, on its tolerogenic effect.
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Keywords: Cholera toxin; Cocoa; Ovalbumin; TCRγδ+ cells; Tolerance

1. Introduction

The intestinal tract is the largest surface of the body protecting the internal toward the external environment. The primary function of the intestine is digestion and absorption of nutrients [1], but it is also recognized as the major immune organ, with the gut-associated lymphoid tissue (GALT) playing a central role in immune system homeostasis [2]. The GALT comprises approximately 70% of immune cells from the entire immune system [2], protecting the enormous intestinal surface (200 m^2 in humans) [3], which is in contact every day with a vast number of potentially harmful antigens [4]. The GALT is structurally and functionally classified into two different compartments: the organized inductive site and the diffuse effector site. The organized compartment is composed of Peyer's patches (PP), mesenteric lymph nodes (MLN) and isolated lymphoid follicles, whereas the diffuse compartment is formed by lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL). PP lymphocytes (PPL) and MLN lymphocytes (MLNL) are considered to be responsible for inducing oral tolerance or initiating immune response to antigens [5,6]. Situated in the lamina propria, LPL also contribute to oral tolerance, respond to antigen uptake and initiate migration of dendritic cells (DC) to the MLN [7]. Located between the epithelial cells, IEL regulate intestinal homeostasis, defend against infection and protect the integrity of the epithelial barrier [8,9].

Food allergies are currently considered a major public health concern due to their increasing prevalence. Food allergies affect approximately 5% of the general population and 8% of children worldwide [10]. According to current understanding, multiple pathways, cells and molecules are involved in the generation of an

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allergic response. Upon oral sensitization, allergens resisting denaturation and digestion in the gastrointestinal tract [11] reach the intestinal epithelium in an intact form and penetrate the first barrier of defense, the mucus layer, produced by the goblet cells and then the intestinal epithelial cell barrier. Afterwards, DC, mainly found in PP, acting as antigen-presenting cells, pick up the allergen and present it to T helper (Th) cells in PP or MLN [12]. Later, Th cells proliferate and differentiate into Th1, Th2, Th17 or regulatory T (Treg) cells according to different cytokine patterns [13]. In most food allergies, an imbalance is evident toward Th2 response [14]. The immune response ends with the activation of effector cells such as B cells, which later turn into IgA-secreting cells. Nevertheless, it still remains a matter of debate which cells are the driving forces for initiating sensitization in the gut.

In a healthy immune response, ingestion of food proteins results in the development of oral tolerance, that is, the suppression of an immune response [7,10]. This immune unresponsiveness affects different immune compartments and is associated with the suppression of antibody production [7]. In contrast, a food allergy develops when there is either a failure in the induction or a breakdown of oral tolerance [15,16]. Due to its increasing frequency, researchers worldwide are focusing on new food allergy preventive measures with increasing awareness of a potential beneficial role of nutraceuticals.

Previous studies have indicated the ability of a cocoa-enriched diet to influence the GALT functionality in rats. Accordingly, we have shown that cocoa consumption modifies the PPL composition in rats [17,18]. The tolerogenic influence of cocoa in a rat oral sensitization model has recently been demonstrated [19]. A 10% cocoa-enriched diet inhibited the synthesis of serum specific anti-ovalbumin (OVA) antibodies and attenuated intestinal IgA. In addition, this nutritional intervention induced changes in the lymphocyte composition and gene expression of MLN [19]. Specifically, in MLN, a cocoa diet increased the proportion of TCRγδ+ cells, playing a crucial role in the tolerance to oral antigens and CD8+ CD103+ cells, associated with regulatory functions. A decrease in CD4+CD62L+ and CD8+CD62L+ cell percentage was additionally observed, indicating a reduced influx of lymphocytes in MLN [19]. Together, these results show the capacity of a cocoa diet to induce oral tolerance and its potential role as a nutraceutical in food allergies.

Despite these previous studies, the influence of cocoa at the intestinal level remains unknown. We hypothesized that cocoa-enriched diet might regulate intestinal lymphoid tissue because it is the first compartment of the immune system in contact, and these changes could thereby contribute to the avoidance of the oral sensitization in rats. On the basis of this hypothesis, the present study aimed to analyze the effects of a cocoa diet on the small intestinal immune system in a rat oral sensitization model. Therefore, intestinal samples from rats orally sensitized with OVA were evaluated for lymphocyte composition in three functional compartments of the small intestinal immune system (PP, IEL and LPL) and also for the effect of a cocoa diet on representative molecules produced by GALT.

2. Materials and methods

2.1. Chemicals, reagents and diets

Albumin from chicken egg white (OVA; grade V), cholera toxin (CT), collagenase, 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), Hanks balanced salt solution (HBSS), Roswell Park Memorial Institute (RPMI), Mayer's hematoxylin solution, eosin Y solution, Percoll®, Trizma® base (Tris Base) and Tween 20 were purchased from Sigma-Aldrich (Madrid, Spain). Donkey serum was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). NaN_3, p-formaldehyde, tri-sodium citrate dihydrate and citric acid were provided by Merck (Darmstadt, Germany) and RNAlater® by Ambion (Applied Biosystems, Austin, TX, USA). Xylene and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Vienna, Austria) and ethanol absolute from VWR (Vienna, Austria). Fluoromont-G was provided by SouthernBiotech (Birmingham, AL, USA). Ketamine was obtained from Merial Laboratories S.A. (Barcelona, Spain) and xylazine from Bayer A.G. (Leverkusen, Germany). Natural Forastero cocoa was obtained from Idilia Foods SL (formerly Nutrexpa S.L., Barcelona, Spain) and AIN-93 M diet and basal mix from Harlan Teklad (Madison, Wisconsin, USA).

2.2. Animals and experimental food intervention

Female Lewis rats were obtained from Janvier Labs (Saint Berthevin Cedex, France) and maintained under conditions controlled for temperature and humidity in a 12-h light/dark cycle. The present experimental design and procedure were approved by the local Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref.5988).

After a week of acclimatization at the Faculty of Pharmacy animal facilities (UB), 3-week-old rats were housed in cages (three per cage) and given *ad libitum* access to water and solid food during the 28 days of the study. The rats were randomly distributed into four experimental groups: reference group (RF/R), reference group fed cocoa diet (RF/C), OVA-sensitized group (OVA/R) and OVA-sensitized group fed cocoa diet (OVA/C) (n=9 each). The RF/R and the OVA/R groups were fed a standard diet (AIN-93 M), whereas the RF/C and the OVA/C groups were fed an isoenergetic diet containing 10% cocoa (Table 1). The OVA/R and OVA/C groups were orally sensitized as described [19,20], receiving 50 mg of OVA plus 30 μg of CT in 1 mL of distilled water by oral gavage three times per week (days 0, 2, 4, 7, 9, 11, 14, 16, 18 and 21). The RF/R and OVA/R groups received 1 mL of distilled water accordingly.

2.3. Sample collection and processing

On day 28, the animals were euthanized, and the small intestine was carefully collected. After discarding the duodenum, the intestine was rinsed with phosphate buffer saline (PBS) solution in order to remove fecal content. A 0.5 cm portion of the middle of the intestine was immediately conserved in RNAlater®, and the consecutive following segment was placed in cassettes and fixed in 4% paraformaldehyde. The rest of the intestine was opened lengthwise along the mesenteric line; PP were collected and stored in RPMI medium. Finally, the remaining intestine was cut into 2 cm pieces and immersed in HBSS supplemented with 10% heat-inactivated FBS to isolate IEL and LPL.

2.4. Peyer's patches lymphocyte isolation

PP were incubated with 1 mM DTT in RPMI medium under continuous agitation (55 u/min, 5 min, 37 °C). Consecutively, DTT medium was discarded, and PP were washed and passed through a sterile 70 μm mesh with a syringe plunger. The suspension obtained was centrifuged (538 *g*, 5 min, 4 °C) and resuspended with RPMI-10% FBS medium. Thereafter, cells were counted and viability was determined by staining with trypan blue using a Countess™ Automated Cell Counter (Invitrogen, Thermo Fisher Scientific).

2.5. Intraepithelial and lamina propria lymphocyte isolation

IEL and LPL isolation was carried in accordance with previous studies [21,22]. Briefly, small pieces of intestine, without PP, were incubated with a 5 mM DTT solution

Table 1
Composition of the experimental diets

Components	Standard diet [1]	10% cocoa diet [2]	
		Basal mix	Cocoa powder
Proteins	140.73	118.27	23.05
Lipids	38.71	27.06	11.53
Carbohydrates	721.93	692.41	16.76
Soluble fiber	–	–	8.91
Insoluble fiber	50.00	24.52	26.72
Minerals	35.86	27.83	6.29
Vitamins	10.20	7.92	0.04
Choline bitartrate	2.56	1.98	–
Antioxidant	0.01	0.01	–
Theobromine	–	–	2.50
Phenolic compounds [3]	–	–	4.02
Total	1000.0	1000.0	

All values are expressed as g/kg of diet.
[1] AIN-93 M formula was used as standard diet.
[2] The 10% cocoa diet was prepared from a basal mix in which 100 g cocoa/kg was added.
[3] Reversed-phase high-performance liquid chromatography coupled to a diode array detector revealed that cocoa phenolic compounds were epicatechin (2.34 mg/g), catechin (0.4 mg/g) and procyanidins.

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