Anaerobe 26 (2014) 24–30

Contents lists available at ScienceDirect

Anaerobe

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

Clinical microbiology

Exopolysaccharide-producing *Bifidobacterium animalis* subsp. *lactis* strains and their polymers elicit different responses on immune cells from blood and gut associated lymphoid tissue

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form
20 December 2013
Accepted 1 January 2014
Available online 18 January 2014

Keywords:
Exopolysaccharide
Bifidobacteria
PBMC
GALT
Immune response
Cytokine

ABSTRACT

The effect of exopolysaccharide (EPS) producing bifidobacteria, and the EPS derived thereof, on the modulation of immune response was evaluated. Cells isolated from gut associated lymphoid tissue (GALT) and from peripheral blood mononuclear cells (PBMC) of naïve rats were used. The proliferation and cytokine production of these immune cells in the presence of the three isogenic *Bifidobacterium animalis* subsp. *lactis* strains (A1, A1dOx and A1dOxR), as well as their purified polymers, were *in vitro* analysed. The cytokine pattern produced by immune cells isolated from GALT showed that most levels remained stable in the presence of the three strains or their corresponding polymers. However, in PBMC the UV-inactivated bacteria induced higher levels of the ratios IFNγ/IL-17, TNFα/IL-10 and TNFα/TGFβ, and no variation in the ratio IFNγ/IL-4. Thus, *B. animalis* subsp. *lactis* strains were able to activate blood monocytes as well as T lymphocytes towards a mild inflammatory Th1 response. Furthermore, only the EPS-A1dOxR was able to stimulate a response in a similar way than its EPS-producing bacterium. Our work supports the notion that some bifidobacterial EPS could play a role in mediating the dialog of these microorganisms with the immune system. In addition, this study emphasizes the effect that the origin of the immune cells has in results obtained; this could explain the great amount of contradiction found in literature about the immunomodulation capability of EPS from probiotic bacteria.

1. Introduction

Bifidobacterium – a strict anaerobic Gram-positive, with high G + C content genus – is a common member of the human intestinal microbiota [1], being the main resident of the infant gut [2]. Positive effects have been attributed to specific strains of this genus and therefore they are considered as probiotics, i.e, "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" [3]. Nowadays *Bifidobacterium*, together with *Lactobacillus*, constitute the main genera used for human applications due to the long history of their safe use in foods [4]. Some ways of probiotics action on health could be summarized as (i) antagonism against pathogens, (ii) enhancement of intestinal barrier function, and (iii) modulation of the host immune response [5]. It is well established that orally ingested probiotics, as well as the commensal microbiota, are able to simulate both innate and adaptive immune responses [6,7]; however, the mechanisms of action are not totally understood. The dialog between probiotics and host is mainly mediated by surface components of bacteria able to interact with specific receptors located on the eukaryotic cells [1,5,8–10]. Bacterial exopolysaccharides (EPS) are one of the surface molecules that act as mediators of this cross-talk. Indeed, some of these carbohydrate polymers are key player in the development of tolerance to commensal microbes, such as the polysaccharide A (PSA) synthesized by *Bacteroides fragilis* [11].

The biosynthesis of EPS has been described as well in several species of *Bifidobacterium*, and it seems that these polymers could play a relevant role in the immune modulation capability of the producing strain [12,13]. It seems that the physicochemical characteristics of EPS, i.e. monomer composition, presence or charged

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http://dx.doi.org/10.1016/j.anaerobe.2014.01.003

substituents and molecular weight, are determinants in the modulation of the immune response. Indeed, negative-charged bifidobacterial EPS seem to be able to activate different immune cells, whereas neutral and high molecular weight (MW) polymers elicit a suppressor profile [13]. These observations have been previously found with other microbial polymers, such as for example those of the fungi kingdom; the acid polysaccharide purified from the mycelia of *Cordyceps sinensis* is able to stimulate macrophages [14], whilst the size of the EPS produced by *Crytococcus neoformans* is inversely correlated with its immunomodulatory ability [15].

In a previous work of our group, 17 polymers purified from bifidobacteria of human intestinal origin have been screened for their immune modulation potential upon peripheral blood mononuclear cells (PBMC) isolated from humans. Results obtained were highly dependent on the intrinsic characteristics of the polymers, although these bifidobacterial EPS were not able to trigger a strong immune response [16]. Of particular interest was the case of the polymers synthesized by three closely related (isogenic) *Bifidobacterium animalis* subsp. *lactis* strains named A1 (parental), A1dOx (derivative from A1 adapted to bile salts) and A1dOxR (obtained from A1dOx after successive cultures which spontaneously acquired a "ropy" phenotype). The strain A1dOxR was, out of the three, the only one able to synthesise a polymer of high-MW ($\sim 10^6$ Da) [17], and this was the polymer showing lower capability to induce the release of cytokines by human PBMC *in vitro*. Therefore, in the current study we aimed to further study this model of EPS-producing bifidobacteria and their polymers. Indeed, we want to address whether the response that the strains elicit at systemic level could predict the response that occur at the intestinal mucosa, the first contact point of ingested probiotics. For that purpose we have *in vitro* exposed immune cells, isolated from blood (PBMC) and from the gut associated lymphoid tissue (GALT) of naïve rats, to these EPS-producing bifidobacteria and their polymers.

2. Materials and methods

2.1. Bifidobacteria strains and culture conditions

The EPS-producing strains *B. animalis* subsp. *lactis* A1, A1dOx and A1dOxR, formerly described, were used in this study. Bifidobacteria were routinely grown in MRSC broth, composed of MRS (Biokar Diagnostics, Beauvois, France) plus 0.25% L-cysteine (Sigma-Chemical Co., St. Louis, MO, USA), at 37 °C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) in the anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK). Stocks of these strains in MRSC + 20% glycerol are held in the IPLA-CSIC collection. As standard procedure, the stock of each strain was plated in the surface of agar-MRSC and an isolated colony picked up to inoculate 10 mL MRSC broth which was incubated overnight. This culture was used to inoculate fresh MRSC broth which was grown for 24 h.

For co-cultivation with immune cells, bifidobacteria cultures were inactivated by UV-radiation as previously described [18]. In short, bifidobacteria were harvested by centrifugation, washed with PBS and the number of viable bacteria was determined by counting in agar-MRSC. Bifidobacteria suspensions in PBS were treated in a UV-chamber (15 W, Selecta, Barcelona, Spain) during three cycles of 30 min each. Finally, the UV-treated bacteria were distributed in aliquots, frozen in liquid N_2 and stored at −80 °C.

2.2. Exopolysaccharide isolation

For the purification of the EPS synthesised by each bifidobacteria, strains were grown in the surface of agar-MRSC for at least 3 days and the biomass was collected using 2 ml ultrapure water per plate. Then, to favour the release of the bound EPS 1 volume of 2 N NaOH was added to the bacterial suspension, which was kept under constant stirring for 16 h at room temperature. After centrifugation, the EPS in the supernatant was precipitated using 2 volumes of chilled absolute ethanol for 48 h at 4 °C. The precipitated EPS was collected by centrifugation, resuspended in water and dialysed (in cellulose membrane 12–14 kDa MWCO, Sigma) against ultrapure water for 3 days at 4 °C. Finally, the dialysed fraction was lyophilised to obtain the EPS powder [19]. Additionally, the polymer purified from the strain A1dOxR was further purified to obtain the high molecular weight (HMW)-EPS-R1 fraction as previously described [17]. In this procedure, the polymer A1dOxR was dissolved in water at 2.5 mg mL^{-1} and dialysed in a Spectra/Por Float-A-Lyzer tube of 100 kDa MWCO (Sigma) for 72 h at 4 °C against ultrapure water with a daily change of water. Finally, the content of the tube was collected and freeze-dried. The protein content of each EPS fraction was determined using the BCA protein assay (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

2.3. Isolation of immune cells from rats

This study was approved by the Animal Experimentation Ethical Committee of the University of Belgrade (Serbia) and by the Bioethical Committee of CSIC (Spain), strictly following the International Directives. A statistically representative number of eight male Wistar rats of the same litter (5–6 weeks old), were purchased from the Farm of the Military Medical Academy, Belgrade and for research purposes were housed in the animal facility at the Faculty of Pharmacy, University of Belgrade. Animals were grown under standard conditions (23–25 °C and 12/12 light-dark cycle) with a commercial diet hiving unlimited access to standard rat food and tap water.

For the experiments each animal was anaesthetized with CO_2 and, once assured the loss of corneal reflex, its intestine was excised from the jejunum to the ileocaecal junction. The whole small intestine was placed in cold Hank's balanced salt solution (HBSS no calcium, no magnesium) and kept at 4 °C until processing. A blood sample (about 2–3 mL) was taken into tubes with the anticoagulant heparin. Finally, the animals were killed by cervical dislocation.

2.3.1. Peripheral blood mononuclear cells (PBMC)

The PBMC from the eight rats were obtained using standard procedures [16]. Briefly, heparinized blood was diluted with 1 volume of Dulbecco's PBS (Sigma) and 6 mL carefully put on top of 3 mL fycoll (Histopaque-1077, Sigma). The gradient was made by centrifugation (500×*g*, 30 min, break-off) and the PBMC phase was aseptically removed with a Pasteur pipette. The isolated PMBC were washed ones with Dulbecco's PBS, counted in a Neubauer chamber (Brand, VWRI Eurolab, Barcelona, Spain) and resuspended at 2×10^6 cells mL^{-1} in RPMI-1640 (with 2 mM L-glutamine, 25 mM Hepes) supplemented with 10% heat inactivated foetal serum bovine and antibiotics (50 μg mL^{-1} penicillin, streptomycin, and gentamicin, each). The medium and supplements were purchased from Sigma.

2.3.2. Cells from gut associated lymphoid tissue (GALT)

The isolation of the immune cell subsets from the rat intestines was performed using a modification of the procedure described by Solano-Aguilar and co-workers [20] as follows (Fig. 1). The whole rat intestine was placed into a petri dish with HBSS solution, cut into small (around 5 cm) pieces and cleaned from its content with a spatula. Several changes of petri dish and cleaning steps were made in order to ensure the maximum removal of the luminal intestinal content. These pieces were longitudinally cut with a blade,