応用薬理 Ōyō Yakuri
Pharmacometrics **93** (5/6) 103-107 (2017)

The Role of Supplement EF-2001 Containing *Enterococcus faecalis* on Murine Ileal Immune Responses *Ex Vivo*

Takeshi Tadano [1),2)]*, Shigetoshi Tsuzuki[2)], Yukio Nemoto[2)], Osamu Nakagawasai[3)], Koichi Tan-No[3)], Hiroshi Onogi[4)], Hiroaki Nishijima[5)], and Hiroyuki Iwasa[6)]

[1)] *Department of Complementary and Alternative Medicine Clinical Research and Development, Graduate School of Medicine Sciences, Kanazawa University, Takara-machi,13-1 Kanazawa Ishikawa 920-1192, Japan,*

[2)] *Kampo and Herbal Medicine Research Center, Yokohama University of Pharmacy, 601 Matano-cho,* Totuka-ku, Yokohama,Kanagawa, 245-0066,Japan,

[3)] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, 981-8558, Miyagi, Japan,*

[4)] *Faculty of Health Science, Tohoku Fukushi University, 1-8-1 Kunimi, Aoba-ku, Sendai 981-8522, Miyagi, Japan,*

[5)] *Department of Healthcare and Regulatory Sciences, School of Pharmacy, Showa University, 1-5-8 Hatanodai, Sinagawa-ku, Tokyo142-8555,Japan*

[6)] *Nihon Berum Co.Ltd, 2-14-3 Nagata-cho, Chiyoda-ku, Tokyo100-0014, Japan.*

Received December 22, 2017, Accepted January 27, 2018

The intestinal flora and Peyer's patches (PP) play an important role in increasing immunity, and ingestion of lactic acid bacteria is considered beneficial. We therefore studied how *Enterococcus faecalis* EF-2001 effects on the regulation of intestinal immunity, by using mouse PP cells with or without concanavalin A (Co A). Production of the T helper 1 (Th1), cytokine IL-2 in cultured IL-2 in culture PP cells co-stimulated with Con A was significantly decreased in mice administered with EF-2001 at dose of 300 mg/kg/day for five consecutive days. In addition, the production of interferon (IFN)-γ, also attributed to Th 1 cytokines, was dramatically enhanced by EF-2001 administration at the same doses. However, EF-2001 did not alter the production of the T helper 2 (Th 2) cytokines such as IL-4 and IL-5 in the same treatment. Further, PP cells from mice administered with EF-2001 significantly produced B cell immunoglobulins, IgG1 and IgA, co-stimulated with lipopolysaccharide LPS. Taken together, we suggested the possibility that EF-2001 modulates a gut immune response especially on B cell and Th 1, and contributes to maintaining homeostasis.

Keywords: *Enterococcus faecalis*/EF-2001/lactic acid bacteria/ileal immune responses /intestinal flora/Peyer's patches

Introduction

From the viewpoint of preventive medicine, it is highly essential to maintain immunity during pre-symptomatic treatment; and lactic acid bacteria help accomplish this. The intestinal flora and Peyer's patches (PP) play an important role in increasing immunity, and ingestion of lactic acid bacteria is considered beneficial. Lactic acid bacteria include bacilli (*Lactobacillus*, *Bifidobacterium*, and *Spirolactobacillus* spp.) and spherical or oval cocci (*Lactococcus*, *Enterococcus*, and *Pediococcus* spp.). These synthesize short-chain fatty acids such as butyric acid, thereby maintaining the tendency towards an acidic state within intestinal tract, and they induce the secretion of the antimicrobial substance α-defensin, which prevents the survival of pathogenic bacteria (Ayabe et al., 2000). Lactic acid bacteria are classified as probiotics, prebiotics, and biogenics. The best known of these, probiotics, have been reported to improve the intestinal environment by adhering to the intestinal flora (Carlson and Gothefors., 1975; Johansson et al., 1993; Fujiwara et al., 2001) however, probiotics are required to be ingested in large doses because they are mainly killed by gastric acid (Mitsuoka, 2014). Prebiotics include oligosaccharides and edible fiber, which act to increase the number of beneficial bacteria. Biogenics act to increase beneficial bacteria and produce IgA from PP cells (Suzuki et al., 2004).

Regarding *Enterococcus faecalis* EF-2001, which has previously been classified as a biogenic, with repeated administration of EF-2001 to mice after destruction of the intestinal flora along with simultaneous administration of

*Corresponding author: Takeshi TADANO, Ph.D.
Graduate School of Medicine Sciences, Kanazawa University
Tel: +81-76-265-2147 Fax+08-76-234-4247
E-mail: tadano@staff.kanazawa-u.ac.jp

the antibiotics cefalexin and ampicillin, early appearance of beneficial *Lactobacillus* and *Bifidobacterium spp.* was noted, suggesting that EF-2001 contributes to improving the intestinal environment (Shimohashi et al., 2002). Moreover, when mice with immunity lowered by cyclophosphamide administration were infected with the opportunist microbes *Candida albicans* and *Pseudomonas aeruginosa*, all of them died. However, when mice infected with these opportunistic organisms after repeated administration of EF-2001, 20% of them survived for prolonged periods (Satonaka et al., 1996). This hypostatized that EF-2001 may enhance immune function mediated by neutrophils and macrophages.

To further clarify details of the immune function of EF-2001, in this study, *Enterococcus faecalis* strain EF-2001 with only the effective components of the cell wall extracted after heat-killed was studied for its effects on the regulation of intestinal immunity, using PP cells from mice.

Materials and methods

Animals

Male 5-week-old C57BL/6N mice (Charles River Laboratories, Japan, Inc.) were used in the experiments. Each data are expressed as the mean±S.E.M. for 10 mice /group. The animals were housed under conditions of constant temperature (23±1℃) and humidity (55±5%), on a 12 h/12 h light-dark cycle (light from 7 to 19 to 7 h). All experiments complied with the Guidelines for Care and Use of Laboratory Animals issued by Kanazawa University.

EF-2001 and treatment

Heat-treated *Enterococcus faecalis* EF-2001 (Nihon Berum Co., Ltd.) was used in this experiment. EF-2001 was produced by the process described below. Because bacterial cell wall including polysaccharides and endotoxins strongly enhances the production of tumor necrosis factor (TNF)-α through the activation of macrophages and cause non-specific immune response in the whole body,; we therefore monitored TNF-α production and consequently, ca. 80% of the activity was detected in the cell wall. Accordingly, the effective components of the cell wall were recovered after activating the cell wall constituents of EF-2001 by heating, and are marketed in a granular form (Ishijima et al., 2014). Moreover, in order to ensure the immunological capacity of EF-2001, EF-2001 is commercialized as a product having anticancer activity of at least 70% as opposed to 100% for the immune activity (anticancer activity) of the anticancer agent polysaccharide Picibanil. In fact, to date various results supporting immunopotentiation have been obtained with ingestion of EF-2001 in human and rodents (Choi et al., 2016; Choi et al., 2016). EF-2001 was suspended in sterilized water and administered with orally once a day for five consecutive days.

Preparation of Peyer's patch (PP) cells

Mice were killed with an overdose of ether. Their small intestines were removed and placed into a petri dish (60-min diameter) on ice, which was filled with ice-cold phosphate-buffered saline (PBS) containing penicillin (PC, 100 U/mL) and streptomycin (ST, 100 μg/mL) (Takano *et al.*, 2003,Takano *et al.*, 2007, Kato *et al.*, 2011). Visible PPs were carefully dissected from the wall of the intestine using micro-scissers under a dissecting microscope (7-11 PPs were obtained from each mouse). These PPs were placed in ice-cold RPMI-1640 medium containing 5% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 50 μM 2-mercaptethanol, 100 μg/mL PC, and 100 μg/mL ST. To obtain a single-cell suspension, individual, PPs were digested with type-I collagenase (70 U/mL, Sigma-Aldrich, St Louis, MO, USA) dissolved in RPMI-1640 medium containing 5% FBS and incubated for 90 min at 37 ℃. After filteration through 70-um nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA). PP cells were washed three times with PBS. Cell viability was assessed by trypan blue exclusion. Morphological analysis by characteristic nonspecific esterase and Giemsa strainig revealed that >95% of cells were lymphoid, <3% were monocytes and -2% were other under cells from ileal tissue. PP cells were seeded in 96-well flat-bottomed plates (Becton Dickinson) and cultered with or without 5 μg/mL concanavalin A (Con A) or 10 μg/mL lipopolysaccharide (LPS) for 48-120 h.

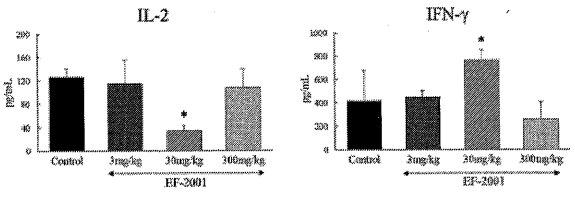

Fig. 1. Effects of EF-2001 on Th1 cytokine production.

PP cells from mice administered with EF-2001were co-stimulated with Con A for 48-72 h. Supernatants were collected and measured Th1 cytokines by using ELISA kit. Data are expressed as the means ± S.D. of sextuplicate cultures.* P<0.05 vs. control group.

Measurement of Th1 and Th2 cell cytokines in PP cell cultures

The culture supernatants in PP cells from mice administered with samples were collected at 48 to 72 hrs after stimulation of Con A, and Th 1 cytokines, IL-2 and IFN-γ, and Th 2 cytokines, IL-4 and IL-5, were measured using a commercial enzyme-linked immunosorbant assay (ELISA) kit (eBioscience) by reading the absorbance of a fluorescent dye, and production of the respective cytokines was calculated from calibration curves for the reference substances.

Measurement of immunoglobulins (IgA and IgG1) in PP cell cultures

EF-2001 (30 mg/kg) was administered to mice once daily for five consecutive days. The PP cells were cultured for 140 hrs in the presence or absence of lipopolysaccharide (LPS; Sigma-ardrich, E.cpli 055B5), and IgA and IgG1 production in the culture supernatant was evaluated using commercial ELISA kit (Bethyl Lab. Inc. Montogomery, TX, USA).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The significance of differences was determined by a one-way analysis of variance (ANOVA), followed by Fisher's PLSD test. $p<0.05$ represented a significant difference.

RESULTS

Effects of EF-2001 on the production of Th cytokines

After repeated administration of EF-2001 at doses ranging from 3 to 300 mg/kg/day, their effects on Th cytokines production in PP cells were investigated. As illustrated in Figs 1 and 2 the production of the Th1 cytokine IL-2 was significantly decreased in mice administered EF-2001 (30 mg/kg). In contrast, the same Th1 cytokine IFN-γ was significantly increased in the group administered EF-2001 at higher dose of 300 mg/kg (Fig. 1). EF-2001 at the same dose did not affect the production of the Th2 cytokines IL-4 and IL-5 (Fig. 2).

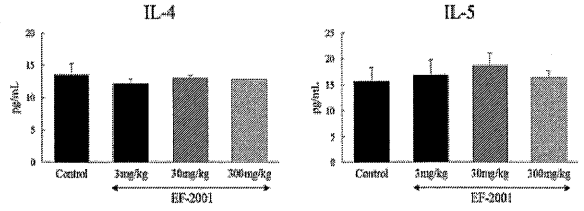

Fig. 2. Effects of EF-2001 on Th2 cytokine production.

PP cells from mice administered with EF-2001were co-stimulated with Con A for 48-72 h. Supernatants were collected and measured Th2 cytokines by using ELISA kit. Data are expressed as the means ± S.D. of sextuplicate cultures.* $P<0.05$ vs. control group.

The effect of EF-2001 on Immunoglobulin IgA and IgG1 production

In the experimental groups using the *Lactobacillus spp. Lactobacillus brevis and Lactobacillus casei as positive* controls, PP cells from mice administered with EF-2001 or *Lactobacillus casei* at dose of 30 mg/kg could produce the IgG1 triggered by LPS. An increment in IgA production was noted owing to EF-2001 administration, but not by *Lactobacillus brevis* or *Lactobacillus casei* at 30mg/kg (Fig. 3).

DISCUSSION

To investigate the phenomena underlying the enhancement of immune responses by *Enterococcus faecalis* EF-2001, we studied its effects on ileal immune function in PP cells ex vivo.

PPs are present in the mucous membrane inside the wall of the small intestine, lymphatic tissues arising from lymphoid nodules on the flat surface of the lamina propria mucosae of the small intestine. In a region with an elliptical shape having a diameter of about 0.5 mm, 7~8 elliptical patches are present. They contain many lymphoid nodules; a single PP comprises ca. 20 lymphoid nodules. The M cells of PP, which play an important role in immunity, transport antigenic information by transcytosis from the intestinal lumen to T-cells or B cells, and macrophages in contact with the basal lamina, which take up antigens such as bacteria. Th1 cells well-known to

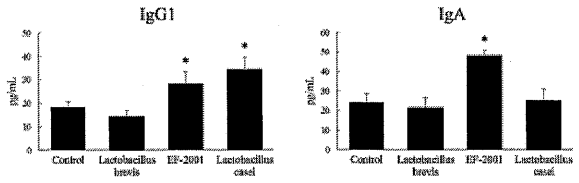

Fig. 3. Effects of EF-2001 on IgG1 and IgA production co-stimulated with LPS.

PP cells from mice administered with EF-2001were co-stimulated with LPS for 96-120 h. Supernatants were collected and measured immunoglobulins IgG1 and IgA by using ELISA kit. Data are expressed as the means ± S.D. of sextuplicate cultures.* $P<0.05$ vs. control group.

produce IL-2, IL-3, and IFN-γ and modulates T cell immune response. In this study, EF-2001 increased the production of IFN-γ among the Th1 cytokines in PP cells stimulated with Con A. This suggests that EF-2001 strengthens host resistance to pathogens and tissue reconstruction via the secretion of IFN-γ. This cytokine reportedly convert unprimed macrophages M1 to armed macrophage M2. However, the fact that IL-2 production by PP cells was suppressed by EF-2001 at the same conditions suggesting the possibility that EF-2001 can defend against autoimmune disorders in the event of a hyperimmune response. EF-2001 did not show any activity with regarding to the induction of Th2 cytokines IL-4 and IL-5.

EF-2001 increased the production of immunoglobulins IgG1 and IgA in PP cells contributed to humoral immune responses. However, this activity was shown in the case of dosage at 30 mg/kg/day. This indicates EF-2001 requires optimal dose which modulates ileal immune responses. The positive control bacteria *Lactobacillus casei*, also enhanced production of IgG1 but not affect IgA. Furthermore, the same bacteria strain *Lactobacillus brevis* affectd neither IgG1 nor IgA. The small intestinal epithelium forms an epithelial barrier layer covered with a thick layer of mucous, and a large quantity of secretory IgA is present at the mucosal surface. IgA is secreted from plasma cells present in the submucosal lamina propria mucosae; in humans, ca. 70% of the plasma cells in the whole body are present in the intestinal tract, and most of these produce IgA (Satonaka *et al.*, 1996). EF-2001 enhanced the production both IgA and IgG1, and IgA production capacity is greater than those induced by *Lactobacillus spp.* This suggests that EF-2001 prevents aggregation of bacteria and viruses of adhesion to the intestinal epithelium and also acts to eliminate or suppress proliferation of microorganisms and brings about neutralization of microbial toxins and dietary antigens. Moreover, bacteria that breach the primary barrier induce mucosal immunity, and secretory IgA antibodies form a secondary barrier that can be expected to contribute to intestinal immunity. Takano *et al.*, (2003) previously reported orally administered the culture fluid of entomogenous fungi *Paecilomyces* into mice did enhance the production of Th cytokines without changing T and B lymphoid population in PP. Further, it also showed that some intestinal bacteria *Lactobacillus* did not alter lymphoid population in PP (Shimohashi *et al.*, 2002). It might therefore be discussed that EF-2001 did not alter lymphoid population in PP by oral administration. Regarding the benefits of EF-2001 in this study, the question of which cell wall constituent(s) affect(s) immune cells such as macrophages, T lymphocytes, B lymphocytes, and/or NK cells is a topic for future study.

In this study we showed the possibility that EF-2001 manifests an immune-activating function when ingested, and contributes to maintaining homeostasis.

Acknowledgement

The authors thank Prof. Dr. F. Takano, Nihon Pharmaceutical University, for his experimental advice.

Disclosures

The authors declare that they have no conflicts of interest to disclose.

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