Contents lists available at ScienceDirect IF: 0.926

Asian Pacific Journal of Tropical Medicine

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

Document heading doi: 10.1016/S1995-7645(14)60148-6

Immune stimulatory activity of BRP-4, an acidic polysaccharide from an edible plant, *Basella rubra* L.

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ARTICLE INFO

Article history:
Received 14 July 2014
Received in revised form 15 August 2014
Accepted 15 September
Available online 20 November 2014

Keywords:
BRP-4
Phagocytic activity
Macrophage
Splenocyte proliferation
Immune stimunlatory

ABSTRACT

Objective: To evaluated the immunomodulatory effect of BRP-4, an acidic polysaccharide from *Basella rubra* (*B. rubra*) L on the macrophage activity. **Methods:** Phagocytic activity was determined by the ingestion of Latex Beads-Rabbit IgG-FITC using the fluorescent microscopy and flow cytometry analysis and nitric oxide production was measured using Griess reaction assay. **Results:** An enhanced production of NO was observed at 10 and 100 μg/mL of BRP-4. The phagocytic activity of macrophage was enhanced in BRP-4 treated RAW264.7 cells. BRP-4 combined with concanavalin A (Con A) provided obvious promotion and strengthening of the proliferation of the splenocytes. **Conclusions:** BRP-4, polysaccharide isolated from *B. rubra*, is suggested to activate macrophage function and stimulate splenocyte proliferation. The strong immunomodulatory activity of BRP-4 confirmed its good potential as an immunotherapeutic adjuvant.

1. Introduction

Plant-based immunomodulators draw many attentions and are investigated for immune modulating activity. Many natural plant products and their derived compounds have been evaluated for their potential to stimulate immune responses[1]. Medicinal plants become an indispensable part of standard health care, based on combination of long-time traditional usage and scientific research. Currently many groups reported the effects of plant-derived polysaccharides on the macrophage activation. Many research groups suggest that the majority of polysaccharides extracted from higher plants, mushrooms and algae enhance host defensive immune responses and promote immunomodulatory, anti-tumorigenic and other therapeutic effects through macrophages[1]. They are promising candidates as immunomodulators with no significant side effects[2]. Polysaccharides from medicinal plants exhibit numerous beneficial therapeutic properties, which presumably are due to the modulation of innate immunity and macrophage function[3,4]. Overall, the primary activity of botanical polysaccharides is to enhance or activate macrophage immune responses, leading to immunomodulatory activity[5]. Numerous dietary polysaccharides, including acidic polysaccharides, appear to possess diverse immunomodulatory activities in various types of animal tissues, including the blood, GI tract and spleen[6].

Basella rubra (*B. rubra*) is a perennial herb and distributed throughout India. It is named as Ceylon spinach, climbing spinach, gui, acelga trepadora, bretana, libato, vine spinach, and Malabar nightshade. In Ayurveda and Folkloric medicine, *B. rubra* L. (Basellaceae) has been used for treating hemorrhages, skin diseases, and sexual weakness and relieving constipation[7,8]. In Chinese traditional medicine, the leaves or the aerial parts of *B. rubra* have been used as relieving constipation and also as a diuretic, a toxicide, and an anti-inflammatory agent[7,9]. The leaves, stems, and young shoot with buds of Basellaceae plant, *B. rubra* L. (Indian spinach) are consumed as a vegetable and health food. BRP-4, isolated from *B. rubra* L., is native

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pectin-type polysaccharides, containing a backbone in which homogalacturonan (HG) regions of -(1→4)-linked D-Gal residues are interrupted by rhamnogalacturonan type I (RG-I) regions consisting of -(1→4)-linked D-galacturonic acid (GalA) residues[10].

Recently, BRP-4 from *B. rubra* was reported to exhibit anti-HSV-2 activity *in vivo*[11]. However, relevant experimental work on the immune stimulatory activity of polysaccharides (BRP-4) isolated from *B. rubra* has not yet been explored. Most of the medicinal food ingredients promote cellular immune responses by increasing lymphocyte proliferation[12] and enhancing macrophage activities[13]. In this study, we investigated the immune stimulatory effect of polysaccharides (BRP-4) from *B. rubra* on macrophage activity and splenocyte proliferation.

2. Materials and methods

2.1. Chemicals and materials

Dulbecco's Modified Eagle's Media (DMEM), fetal bovine serum (FBS) (Gibco/Invitrogen, Carlsbad, CA, USA), Phagocytosis Assay Kit (IgG FITC) (Cayman Chemical Company, Ann Arbor, MI, USA). BRP-4 was kindly provided by Dr. Hayashi's group[10].

2.2. Cell culture

RAW264.7 murine macrophage cells (TIB71) were obtained from the American Type Culture Collection (Manassa, VA). Each cell line was cultured in Dulbecco's Modified Eagle's Media (DMEM; Gibco/Invitrogen, Carlsbad, CA, USA), as previously described[14].

2.3. Determination of nitric oxide

Nitric oxide production was determined as previously described[15] with slight modification. RAW264.7 cells (2×10^5 cells/mL) were cultured in 48-well plates and incubated in the absence or presence with various concentrations (0, 1, 10 and 100 g/mL) of BRP-4. After 20 h incubation, 100 L of the culture medium was mixed with 100 L of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) at room temperature for 10 min. The absorbance at 540 nm was then determined using a microplate reader (Tecan, Männedorf, Switzerland).

2.4. Fluorescent microscopy

Phagocytosis was identified by the Latex Beads-Rabbit IgG-FITC Solution, using the Phagocytosis assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.). RAW264.7 cells (1×10^5 cells/well) were grown on 48-well culture plates. RAW264.7 cells were treated with BRP-4 (0, 1, and 10 g/mL) and then added the Latex Beads-Rabbit IgG-FITC Solution. After 20 h incubation, cells were observed under an inverted fluorescence microscope with eGFP filter (Carl Zeiss, Göttingen, Germany).

2.5. Flow cytometry analysis

RAW264.7 cells (5×10^5 cells/well) grown on 12-well culture plates, incubated in the absence or presence of BRP-4 and then added the Latex Beads-Rabbit IgG-FITC Solution. After harvesting cells, the FITC dextran internalized cells was analyzed by a flow cytometer (FACS calibur, Becton Dickinson) and expressed as mean fluorescence intensity (MFI).

2.6. Experimental animals for primary splenocytes

BALB/c mice (female, adult) were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University and maintained in the Department of Biochemical Science and Technology, College of Life Science, National Taiwan University. The mice were housed and fed a standard Lab diet (normal chow diet) individually. The animal room was kept on a 12-h-light and 12-h-dark cycle. Constant temperature (25±2) ℃ and humidity were maintained. The animals (8- to 10-week old) were sacrificed using CO_2 inhalation to obtain spleens. The abdominal cavities were opened aseptically and the spleens were removed

2.7. Splenocyte proliferation assay

The spleen cell proliferation response was assayed as described previously[16]. Briefly, spleens were removed aseptically from BALB/c mice. Splenocytes were prepared by lysing the red blood cells. Splenocyte proliferation was determined using the method as described by Sun *et al*[17] with some modifications. Briefly, 100 μL of splenocytes at 5×10^5 cells/mL was seeded in triplicate in 96-well microtiter plates, thereafter concanavalin (Con) A (final concentration 3 μg/mL), or medium was added giving a final volume of 200 μL. After 30 min incubation of Con A, cells were incubated with BRP-4 (1 μg/mL) for 48 h. Then, 10 μL of CCK-8 was added to each well and incubated for further 4 h. The absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference.

2.8. Statistical analysis

Results are expressed as mean±standard deviation (SD). Student's *t* test or One-way ANOVA/Dunnett's *t* test was used for assessment of significance between controls and treatments. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of BRP-4 on the NO production in macrophages

RAW264.7 cell line is commonly used in studies of innate immune responses to pathogens[18]. NO is a major mediator of macrophages, which acts as a destroyer to bacteria and tumor cells[19]. To determine the activity of macrophages, the production of NO was measured from the cell supernatant after stimulating the macrophages with BRP-4. The levels of NO were significantly increased in BRP-4 treated RAW264.7 cells (Figure 1). To rule out the possibility of LPS contamination during the purification process, we examined the effect of BRP-4 in the presence of polymyxin B, which neutralizes LPS activities on NO production. Polymyxin B treatment inhibited LPS-induced NO production but not BRP-4 induced NO production (data not shown). Thus, the activity of BRP-4 is not due to the LPS contamination.

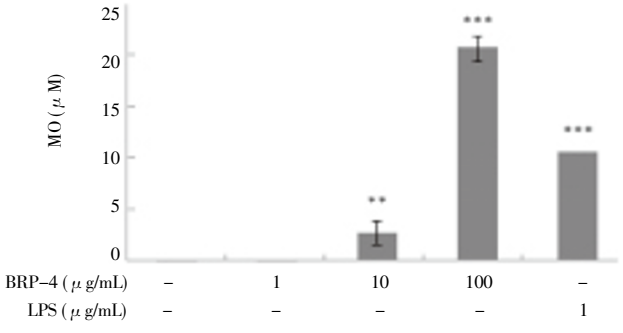

Figure 1. Effect of BRP-4 on the nitric oxide production in RAW264.7 cells.
The culture supernatant was collected to measure the NO production with Griess reagent. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (**P*<0.05, ***P*<0.01, ****P*< 0.001 *vs.* control).

3.2. Effect of BRP-4 on phagocytosis activities of macrophages

Macrophages are considered as the pivotal immunocytes of the host defense. In general, macrophage functions involve phagocytosis of antigens, microorganisms and cellular debris, and killing of invading microorganisms and tumors. To determine if BRP-4 activates immune function, we measured the phagocytic activity of RAW264.7 cells. The phagocytic activity of BRP-4 on macrophage phagocytosis were tested by phagocytosing FITC-expressing (*i.e.*, fluorescence) latex bead. As shown in Figure 2a, BRP-4 treated macrophages internalized much larger numbers of IgG FITC fluorescent latex beads than did untreated cells. Figure 2b and 2c indicate that phagocytosis increased in a dose dependent manner when incubated with BRP-4. These results indicate that BRP-4 stimulates the phagocytic activity against IgG-opsonized particles.

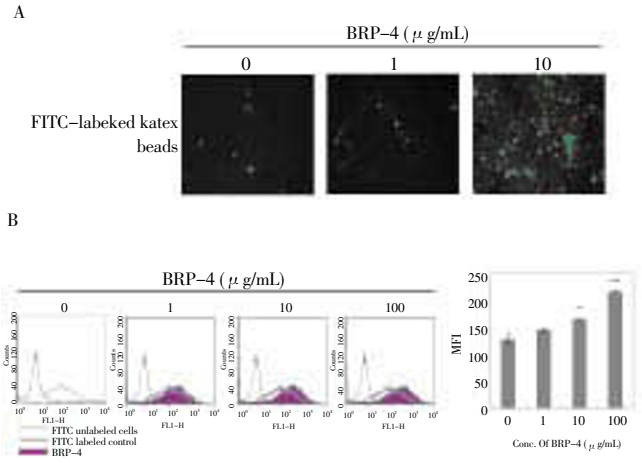

Figure 2. Effects of BRP-4 on phagocytosis in RAW264.7 cells.
(a) Phagocytosis of RAW264.7 cells to the FITC-labeled latex beads was examined after being treated with BRP-4 (0, 1, and 10 g/mL) for 20 h. Figure shown is one representative of three independent experiments (magnification, 100×) under phase-contrast microscopy. (b) Phagocytosis assays were performed after staining of RAW264.7 cells with IgG FITC fluorescent latex beads at the time of opsonization and then subjected to flow cytometric analysis. The histogram overlays are labeled as follows: dot lines, unlabeled cells; gray line, labeled cell in the presence of vehicle (FITC labeled control). Results shown are representative of three experiments. (c) Values were quantified as mean fluorescence intensity (MFI) by flow cytometry. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* FITC labeled control).

3.3. Effect of BRP-4 on splenocyte proliferation

Effects of BRP-4 on splenocyte proliferation with mitogen (Con A) and without mitogen were shown in Figure 3. BRP-4 significantly improved the effect of splenocyte proliferation. After exposure to Con A, BRP-4-treated splenocyte showed significantly higher levels of stimulated proliferation of splenocytes than those in the control group or Con-A group (*P*<0.05 or *P*<0.01). BRP-4 combined with Con A significantly

promoted and strengthened the proliferation of the splenocytes (Figure 3). The present assay showed that BRP-4 could significantly promote the Con A stimulated splenocyte proliferation, which could significantly increase or restore lymphocyte proliferation potential.

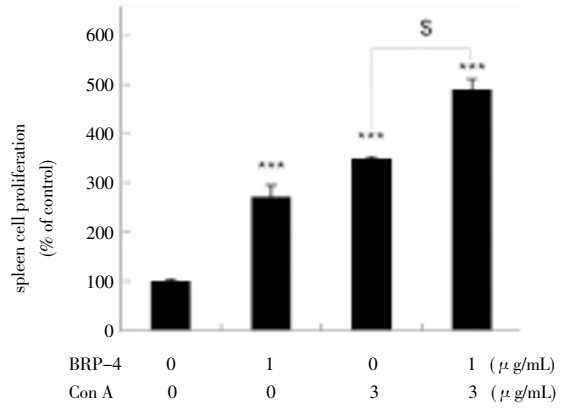

Figure 3. Effects of BRP-4 on proliferation of splenocytes.
Splenocytes were treated with BRP-4 (0 and 1 g/mL) for 48 h after 30min treatment of ConA (3 g/mL) or vehicle. Primary splenocyte proliferation was assessed using CCK-8 assays. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (*P<0.05, **P<0.01, ***P<0.001 vs. vehicle treated control, #P<0.001 *vs*. ConA treated control).

4. Discussion

Polysaccharides from medicinal plants exhibit numerous beneficial therapeutic properties, which presumably are due to the modulation of innate immunity and macrophage function[3,4]. Overall, the primary activity of botanical polysaccharides is to enhance or activate macrophage immune responses, leading to immunomodulatory activity[5]. Numerous dietary polysaccharides, including acidic polysaccharides, appear to possess diverse immunomodulatory activities in various types of animal tissues, including the blood, GI tract and spleen[6]. Thus, we evaluated novel BRP-4, polysaccharides from *B. rubra* that might provide a unique opportunity for the discovery of novel therapeutic agents or adjuvants that exhibit immunomodulatory activities.

Macrophages function as 'pathogen sensors' and performs variety of biological activities, including phagocytosis, chemotaxis, destroying invading pathogens or tumor cells, regulation of the adaptive immune response, and repair of damaged tissue. Activated macrophages markedly enhanced ability to kill and degrade intracellular microorganisms[20]. This killing is caused by an increase of the inducible NO synthase (iNOS) gene to produce NO. It is reported that the amount of NO production by macrophages from old rats in response to Con A decreases compared with that of young rats[21]. Macrophages exhibits cytotoxicity through phagocytosis[1]. They can also function as antigen-presenting cells and can interact with T lymphocytes to modulate the adaptive immune response[22]. Activated macrophages not only participate in both specific and non-specific immune reactions, but also is the "bridge cell" of these two immune reactions[23].

Many studies demonstrated that polysaccharides isolated from medicinal plants or food increased macrophage cytotoxic activity against tumor cells and microorganisms, activated phagocytic activity, increased reactive oxygen species (ROS) and nitric oxide (NO) production, and enhanced the production of cytokines and chemokines, including tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-6, IL-8, IL-12. Previously our group demonstrated that an acidic polysaccharide (APS) was isolated from the extract of Cordyceps militaris grown on germinated soybeans exhibited anti-influenza virus A activity and induce macrophage activation through promoting NO production and cytokine mRNA expression in macrophages[24]. Recently, BRP-4 from *B. rubra* was reported to exhibit anti-HSV-2 activity *in vivo*[11]. Similarly, we observed that BRP-4 stimulated NO production in RAW264.7 cells. The present study investigated the phagocytic activity of murine macrophages. The phagocytic activity of macrophage was enhanced in BRP-4 treated RAW264.7 cells.

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses[25]. Lymphocytes induced by ConA *in vitro* may be used as a method to evaluate T lymphocyte activity[25]. BRP-4 showed a definite and clear synergistic effect on splenocyte proliferation after combining with Con A. It was indicated that BRP-4 could significantly increase the activate potential of T cells. However, the detailed mechanism of its action remained unclear.

In summary, BRP-4 exhibited potent immunopotentiation properties, such as enhancing the phagocytic activity of macrophages and elevating NO production. The splenocyte proliferation was also promoted by BRP-4. Our investigations suggested that BRP-4, polysaccharide from *B. rubra*, has the beneficial effect on immunostimulation. This study may provide an opportunity for developing BRP-4 as an ingredient of functional food and a potential alternative medicine.

Conflict of interest statement

We declare that we have no conflict of interest

Acknowledgements

We, authors, would like to appreciate Dong Ki Park for reviewing the writing and Sun mi Kim from Cell Activation Research Institute (CARI, Seoul, Korea) for assisting detection of phagocytosis activities.

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