

Enhancement of proliferation and differentiation in bone marrow hematopoietic cells by *Spirulina* (*Arthrospira*) *platensis* in mice

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Abstract

This study evaluates whether *Spirulina*, including its components such as phycocyanin, enhances or sustains immune functions by promoting immune competent-cell proliferation or differentiation. The effects of *Spirulina* of a hot-water extract (SpHW), phycocyanin (Phyc), and cell-wall component extract (SpCW) on proliferation of bone marrow cells and induction of colony-forming activity in mice were investigated. The *Spirulina* extracts, SpHW, Phyc, and SpCW, enhanced proliferation of bone-marrow cells and induced colony-forming activity in the spleen-cell culture supernatant. Granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) were detected in the culture supernatant of the spleen cells stimulated with the *Spirulina* extracts. Bone marrow-cell colony formation in soft-agar assay was also significantly induced by the blood samples and the culture supernatants of the spleen and Peyer's patch cells of the mice which ingested *Spirulina* extracts orally for 5 weeks in *in vivo* study. Ratios of neutrophils and lymphocytes in the peripheral blood and bone marrow, consequently, increased in the mice.

Spirulina may have potential therapeutic benefits for improvement of weakened immune functions caused by, for example, the use of anticancer drugs.

Introduction

Spirulina platensis is a helicoidal filamentous blue-green alga (cyanobacterium) and has a history of being used as food for over a thousand years and has been commercially produced for more than 30 years as a food supplement (Ciferri, 1983; Kay, 1991; Belay, 1997). *Spirulina* is known to have nutritional advantages of high-quality protein content and other components such as vitamins; minerals, and essential fatty acids including γ -linolenic acid, and β -carotene (Belay et al., 1993). Recently, more attention has been given to the study of the therapeutic effects of *Spirulina*. In addition to its effectiveness in reducing hyperlipidemia, diabetes and high blood pressure in humans and animals, anti-viral and anti-cancer effects of orally adminis-

tered *S. platensis* involving immune functions have also been reported (Belay, 2002). Previously, we reported that *Spirulina* and its extracts enhanced immune responses in mice, mainly through increased production of interleukin-1 (IL-1) in macrophages (Hayashi et al., 1994, 1998). Phycocyanin, a characteristic photosynthesis pigment protein and an antioxidant in *Spirulina*, has been known to promote the growth of a human myeloid cell line, RPMI 8226 (Shinohara et al., 1988). Recently, Liu et al. (2000) reported that phycocyanin inhibited growth of human leukemia K562 cells and enhanced the arrest of the cell growth at G1 phase, suggesting enhancement of differentiation of the cells. In the mice which ingested phycocyanin for 6 weeks, a marked increase of antigen-specific IgA, as well as total IgA level was observed in the Peyer's patches,

mesenteric lymph nodes and intestinal mucosa, as well as in the spleen cells (Nemoto-Kawamura et al., 2004). These findings suggest that *Spirulina*, and its components such as phycocyanin, affects immune functions by promoting immune competent-cell proliferation or differentiation in lymphoid organs.

In the present study, we investigated effects of *Spirulina* and its extracts on the induction of colony stimulating factor(s) and on their proliferation and differentiation activity for hematopoietic cells in mice.

Materials and methods

Preparation of Spirulina and its extracts

Hot-water extract of *Spirulina* (SpHW): Spray-dried powder of *Spirulina platensis* (30 g) supplied by Dainippon Ink & Chemicals Inc. (Tokyo, Japan) was extracted with 300 mL of boiling water for 1 h. The supernatant of the extract was freeze-dried to obtain a pale blue-green powder (8.22 g) designated as SpHW. SpHW contained 36.3% (w/w) protein and 10% carbohydrate as described before (Hayashi et al., 1994, 1998).

Phycocyanin (Phyc): Phycocyanin was extracted from spray-dried *S. platensis* with 0.05M phosphate buffer (pH 6.0). The resulted crude phycocyanin was dissolved in 0.05M phosphate buffer (pH 6.0) into 4% solution and precipitated twice by 20% and then 60% saturated ammonium sulfate. The resulting precipitate was recovered by centrifugation, dissolved and dialyzed against a 0.05M phosphate buffer (pH 6.0) at 5 °C for 20 h, and applied to a DEAE-cellulose (Whatman DE52) column equilibrated with the same buffer. The phycocyanin fraction was eluted with 0.1M KCl solution. The collected fraction was reprecipitated with ammonium sulfate, dialyzed, and recovered. Phycocyanin content of the preparation (Phyc) was 82–86% from the result of molar absorption coefficient with A_{620} , and the recovery from the crude lyophilized phycocyanin was 6% (Nemoto-Kawamura et al., 2004).

Cell wall components (SpCW): Spray-dried powder (50 g) of *S. platensis* was treated with 0.05N KCl, 1N NaCl solution, and further treated with 0.1% sodium dodecyl sulfate to remove cytoplasmic material. The cell wall preparation was thoroughly washed with distilled water and recovered. Yield from the dried powder was 1.85 g (3.7%).

Bone marrow cells obtained from the femora were used for cell-proliferation and colony forming as-

says measured by fluorometric Alamar BlueTM reduction method (Page et al., 1993) and soft agar method (Metcalf and Foster, 1967), respectively.

The femora were excised from BALB/cA Jcl mice sacrificed by cervical dislocation, and flushed of bone marrow cells using a 27-gauge needle and cold RPMI 1640 (Nikken Biomedical Laboratory, Kyoto, Japan) (Moore et al., 1967). The cells were suspended in RPMI 1640, supplemented with 10% fetal bovine serum (FBS; GIBCO Lab., NY), in a density of 1×10^5 cells 0.2 mL^{-1} well⁻¹ after being washed with the medium, and cultured by using a 96-well tissue culture plate (FALCON 3872, Becton Dickinson Labware, NJ) with or without stimulants such as SpHW, Phyc, and SpCW (1 mg mL^{-1} PBS, $0.02 \text{ mL well}^{-1}$), or culture supernatants (CS, $0.02 \text{ mL well}^{-1}$) of lymphoid-organ cells, for 8 days at 37 °C in a humidified atmosphere of 5% CO₂ in air. The culture supernatants were obtained from the cells cultured with SpHW, Phyc, and SpCW (0.2 mg mL^{-1} final concentration) as described later. 24 h prior to culture termination, $20 \mu\text{L}$ Alamar BlueTM solution (Trek Diagnostic Systems Inc.) was added to each well, and the cells were then continuously cultured. Fluorescence intensity was measured with a Fluoroskan II (Flow Laboratories Inc., USA) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Data were obtained from 6 wells per sample.

For colony forming assay, a plating mixture was prepared in α -MEM (GIBCO Lab., NY), supplemented with 100 IU mL^{-1} penicillin and $100 \mu\text{g mL}^{-1}$ streptomycin, containing final concentrations of 2×10^5 bone marrow cells mL^{-1} , 20% FBS, 0.3% Bacto agar (Difco, MI), and 25% test sample solution. One mL of the mixture was plated into each of two 35 mm diameter wells of a 6-well culture plate (COSTAR #3516, CORNING, NY) and cultured for 7 days at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cell-aggregates composed of more than 50 cells and of less than 50 but more than 20 cells were counted as a colony and a cluster, respectively, under an inverted microscope (Metcalf et al., 1967).

ELISA for GM-CSF and IL-3

Both granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) contents in the culture supernatant or the serum specimens were measured by using commercially supplied assay kits, Mouse GM-CSF ELISA kit (Hbt HK204, HyCult biotechnology, Uden, the Netherlands) for GM-CSF