Journal of Applied Phycology (2006) 18: 47–56
DOI: 10.1007/s10811-005-9014-6

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

Osamu Hayashi[1,*], Shoji Ono[1], Kyoko Ishii[1], YanHai Shi[2], Tomohiro Hirahashi[3] & Toshimitsu Katoh[3]

[1]*Department of Health and Nutrition, Kagawa Nutrition University, 3-9-21 Chiyoda, Sakado 350-0288, Japan;* [2]*Shanxi Cancer Research Center, Shanxi 030013, PR of China;* [3]*Biochemical Division, Dainippon Ink & Chemicals Inc., Ichihara, Chiba 290-8585, Japan*

*Author for correspondence: e-mail: hayashi@eiyo.ac.jp fax: +81-49-282-3718

Received 20 July 2005; accepted 29 November 2005

Key words: cell differentiation, colony-stimulating factor, IL-3, phycocyanin, *Spirulina platensis*

Abstract

This study evaluates whether *Spirulin*a, 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 administered *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 assays measured by fluorometric Alamar Blue™ 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 mL $well^{-1}$), or culture supernatants (CS, 0.02 mL $well^{-1}$) of lymphoid-organ cells, for 8 days at 37 °C in a humidified atmosphere of 5% CO_2 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 μL Alamar Blue™ 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 μg mL^{-1} streptomycin, containing final concentrations of 2×10^5 bone marrow cells mL^{-1}, 2 0% 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% CO_2 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

and Mouse IL-3 ELISA kit (cat. #KMC0030-SBO, COSMO BIO, Tokyo, Japan) for IL-3, respectively, following 2- or 4-fold dilution of each sample, if necessary, according to the instruction manuals, in duplicate. Absorbance at 450 nm of each sample and standard was measured using a microtiter plate reader (Colona Electronics, Japan).

Induction of colony-forming activity

A. in the spleen cells or peritoneal-exudates cells cultured with Phyc, SpHW, and SpCW in in vitro *study*
Spleen cells from C3H/HeJ Jcl mice (6–8 week old, male) were treated with Tris-NH_4Cl solution to remove red blood cells, then washed with Hank's balanced salt solution (HBSS, GIBCO Lab., NY), and finally suspended in RPMI 1640 medium supplemented with 10% FBS (GIBCO Lab., NY). Peritoneal-exudates cells as macrophages were also separated from 5 unstimulated BALB/cA Jcl mice (6-week old, male) and suspended in the same medium as above. Cells were cultured in a density of 1 or 2×10^6 mL^{-1} $well^{-1}$ by using a 24-well culture plate with or without SpHW, Phyc, and SpCW (0.5–4 mg mL^{-1}, 0.1 mL $well^{-1}$) as a stimulant in triplicate for 6 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture supernatant from each well (SpHW-CS, Phyc-CS, SpCW-CS) was collected separately, filtered through a 0.2 μm filter to remove cells and to maintain sterility, and stored at −80 °C until colony assay and cytokine determination. As positive control for colony assay, culture supernatant of spleen cells from BALB/c mice cultured with 0.5% final concentration of PWM (GIBCO Lab., NY) was obtained and designated as PWM CS. PWM is known to induce granulocyte-stimulating factor (G-CSF) in the culture of mouse spleen cells (Metcalf et al., 1967).

B. in the mice fed with or intraperitoneally injected with the Spirulina *extracts in* in vivo *study*
Female inbred C3H/HeN Jcl mice (Clea Japan Inc., Japan) aged 6 weeks were used. Twenty-four mice were divided into four groups (Cont., SpHW, Phyc., SpCW) and kept in a constant temperature (25 ± 1°C) and relative humidity (60 ± 5%) with a 12 h light period from 08:00 to 20:00. Laboratory chow, CE-2 (Clea Japan Inc., Japan) and water were given *ad libitum*. A hot-water extract of *Spirulina*, SpHW, and phycocyanin (Phyc) dissolved in PBS (−) to prepare 5 mg mL^{-1} solution were sterilized by filtration through a 0.22 μm filter. The cell wall component fraction, SpCW, was suspended in 5 mg mL^{-1} PBS (−), and sterilized by autoclaving. One mg of each preparation, SpHW, Phyc, and SpCW, per 0.2 mL per mouse was orally administered to each mouse of the groups once a day and for 5 consecutive days a week via an animal feeding catheter inserted gently into the stomach for 1 or 5 weeks. For the mice of Cont. group, sterilized PBS (−) was administered orally.

The day after the last ingestion, all mice of each group were exsanguinated from femoral artery under anesthesia. An aliquot of the blood from each mouse was used for cell classification and serum sample was separated from the remaining blood for measurement of colony forming activity and CSF content. The peritoneal-exudates (PE) cells were combined from 3 mice of each group as mentioned above by washing the abdominal cavity with HBSS without stimulation. Spleen (SP) cells collected from 3 mice of each group were suspended in HBSS and treated with Tris-NH_4Cl solution to remove red blood cells. Peyer's patches collected were gently homogenized in dispase solution and the resulting (PP) cells were suspended in HBSS. These resulting SP, PP, and PE cells of each lymphoid organ were finally suspended in RPMI 1640 medium supplemented with 10% FBS (GIBCO Lab., NY). The cells prepared were cultured for 6 days in a density of 1 or 2×10^6 cells mL^{-1} $well^{-1}$ by using a 24-well culture plate in triplicate with or without phycocyanin (0.2 mg mL^{-1}) as a stimulant for induction of CSFs. Culture supernatant from each well was collected separately on the last day of culture, filtered through a 0.22 μm filter to remove cells and to maintain sterility, and stored at −80°C until colony assay.

As another preliminary study, *Spirulina* extracts, SpHW, Phyc, and SpCW (10 mg mL^{-1} PBS, 0.5 mL $mouse^{-1}$) were intraperitoneally injected into C3H/HeJ Jcl mice (8 week-old, male), 3 mice per specimen. Sera (Phyc-, SpHW-, and SpCW-serum) were collected 6 h after injection, sterilized by filtration through a 0.22 μm filter, and stored at −80°C until use. Three other mice were treated with LPS (*Escherichia coli* 055:B55, Difco, MI), 100 μg 0.5 mL^{-1} $mouse^{-1}$, to collect the serum (LPS serum) as a positive control for assays. It is known that *i.p.* injection of LPS can induce granulocyte-stimulating factor (G-CSF) in the serum of mice (Metcalf et al., 1967).

The peripheral blood cells and bone marrow cells obtained from the mice of each orally ingested group were morphologically classified by light microscopic observation after treatment with non-specific esterase staining or May-Grünwald-Giemsa staining (Hudson & Hay, 1976).

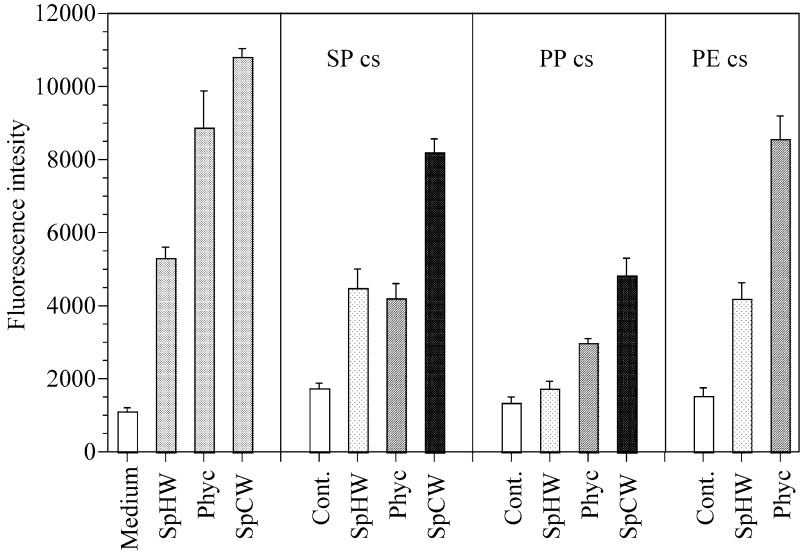

Figure 1. Bone marrow-cell proliferation by *Spirulina* extracts, SpHW, Phy, and SpCW, and by culture supernatant (CS) of lymphoid organ, spleen (SP) and Peyer's patch (PP), and peritoneal-exudates (PE) cells stimulated with the *Spirulina* extracts (values are mean ± SD, n = 6).

Bone marrow cells were also obtained from the femora of each mouse of the groups. Cells were suspended in RPMI 1640 supplemented with 10% FBS in a density of 2×10^5 mL^{-1} and 0.2 mL of the suspension per chamber was centrifuged at 300 rpm for 10 min to collect the cells on a slide glass. Cell morphology was measured under light microscopy after staining, the same as above.

The data in each experiment were statistically analysed by Student's t-test.

Table 1. GM-CSF and IL-3 contents in the culture supernatants (CS) of the spleen cells stimulated with *Spirulina* extracts (values are mean ± SD, N = 3).

Stimulated with	Colonies well^{-1}	GM-CSF pg mL^{-1} of CS	IL-3 pg mL^{-1} of CS
Control	0.5 ± 0.7	<4	47.3 ± 4.0
SpHW	2.8 ± 2.6	<4	76.7 ± 8.0
Phycocyanin	14.0 ± 5.9	9.2 ± 0.7	94.7 ± 10.8
SpCW	28.2 ± 5.5	1,206 ± 333	481.7 ± 144.4

Results

Culture supernatants of spleen (SP), Peyer's patch (PP), and peritoneal-exudated (PE) cells cultured with 20 μg mL^{-1} of *Spirulina* extracts significantly enhanced proliferation of bone marrow cells (Figure 1). Each of the *Spirulina* extracts, SpHW, Phyc, and SpCW, itself, enhanced proliferation of bone marrow cells in the concentration of 100 μg mL^{-1} of culture medium.

Colony- and cluster-formations of the bone marrow cells in existence with culture supernatants of the spleen cells which were stimulated with different final concentrations of *Spirulina* extracts, 50–400 μg mL^{-1}, were measured by soft agar method. Colony- and cluster-formations by the supernatants of cells cultured with Phyc and SpCW were significantly higher than either that of control or of the smallest concentration of each extract (Figure 2A). Culture supernatants of PE cells stimulated with the extracts (200 μg mL^{-1} in final concentration) also enhanced colony- and cluster-formation (Figure 2B). The numbers of these colonies, however, were almost the same as that by culture supernatant stimulated with LPS 10 μg mL^{-1}

The culture supernatants of spleen cells stimulated with SpCW or Phyc, which enhanced colony formation, contained high amounts of GM-CSF as well as IL-3 (Table 1). Culture supernatant of the cells stimulated with SpHW also contained relatively high amounts of IL-3, although colony formation by the supernatant was not so high. GM-CSF and IL-3 in the culture supernatants of PE cells stimulated with SpHW, Phyc, and SpCW were also measured (Table 2). The prepared PE cells consisted of macrophages and lymphocytes in a ratio of about 50% each, and a small ratio of mast cells and neutrophils. The culture supernatant of the cells stimulated with SpCW contained high amounts of GM-CSF but not of IL-3. On the other hand, both

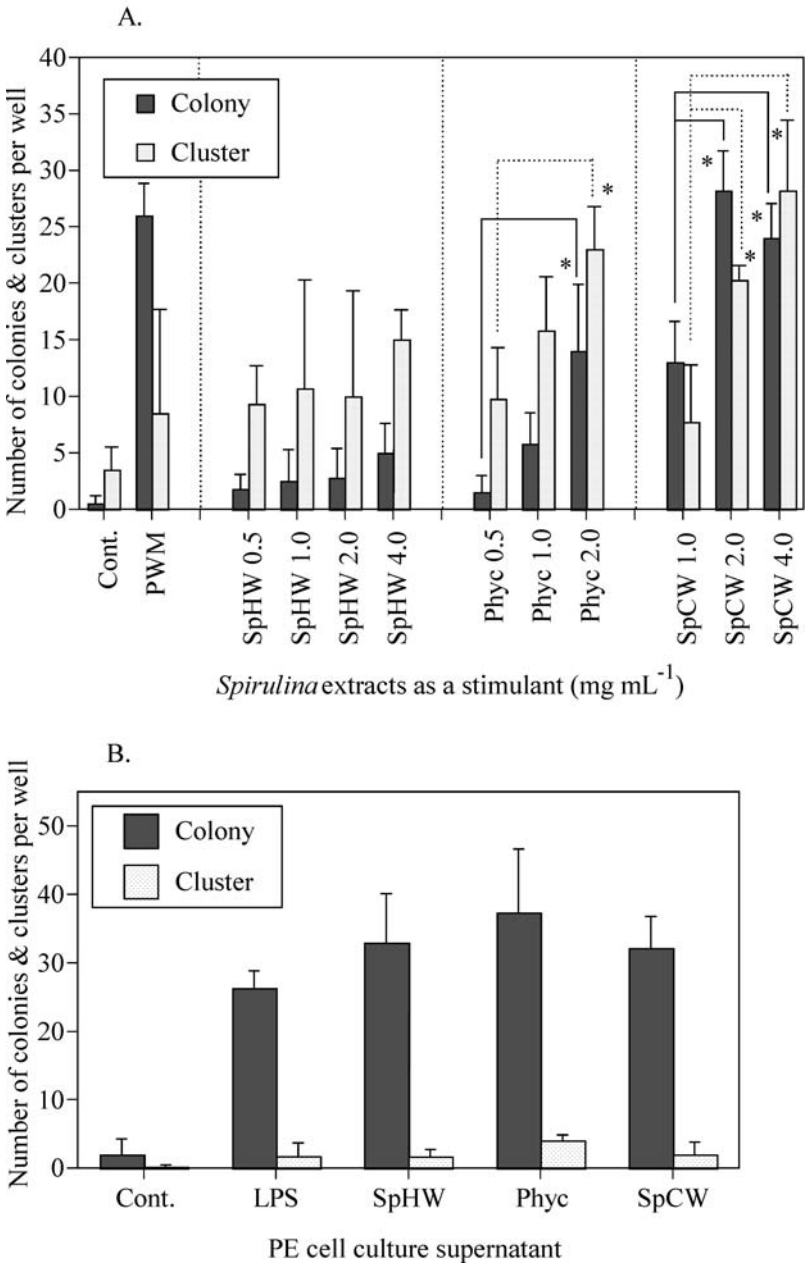

Figure 2. Bone marrow-cell colony and cluster formation in soft agar assay with the culture supernatant of the spleen cells (A) and peritoneal-exudates cells (B) stimulated with *Spirulina* extracts. Spleen cells were stimulated with 0.5, 1.0, 2.0 and 4.0 mg *Spirulina* extract mL^{-1}. Peritoneal-exudates cells were stimulated with 2.0 mg *Spirulina* extract mL^{-1} (values are mean ± SD, n = 3).

SpHW and Phyc induced relatively high amounts of IL-3 in the culture supernatants.

As a preliminary experiment for the next *in vivo* study, we measured colony forming activity in the mice fed with the *Spirulina* extracts for 5 consecutive days, or treated with an intra-peritoneal single injection of the extracts. Figure 3 shows that the serum from the mice which ingested Phyc or SpCW for 5 consecutive days enhanced colony formation of bone marrow cells. Phycocyanin significantly increased the colony formation in comparison to controls, in which normal serum was added. Sera separated from the mice which were treated with intra-peritoneal injection of the *Spirulina* extracts showed generally high colony formation (Figure 4).

Table 2. GM-CSF and IL-3 contents in the culture supernatant (CS) of the peritoneal-exudates cells stimulated with *Spirulina* extracts.

Stimulated with	Colonies well^{-1}	GM-CSF pg mL^{-1} of CS	IL-3 pg mL^{-1} of CS
Cont.	2.0 ± 2.3	<4	<3
SpHW	33.0 ± 7.1	7.1	10.7
Phycocyanin	37.3 ± 9.3	4.3	11.0
SpCW	32.2 ± 4.6	104.7	<3

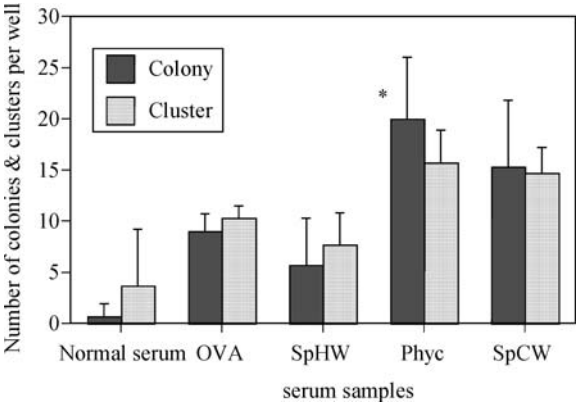

Figure 3. Bone marrow-cell colony and cluster formation in soft agar assay with the serum from the mice fed with *Spirulina* extracts for 5 consecutive days (values are mean ± SD, n = 3).

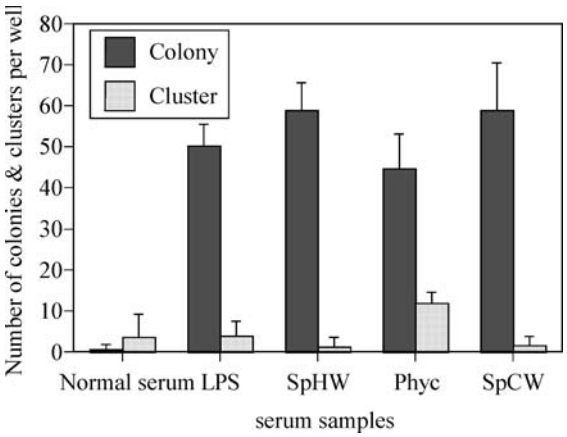

Figure 4. Bone marrow-cell colony and cluster formation in soft agar assay with the serum from the mice intraperitoneally injected with *Spirulina* extracts (values are mean ± SD, n = 3).

The numbers of colonies formed by the sera, however, were almost the same as those formed by LPS serum used as a positive control. Colony-stimulating factors, GM-CSF and IL-3, in the sera from the mice which were either fed or intra-peritoneally injected with the extracts, however, were under detection limit (<4 and 3 pg mL^{-1} serum, respectively). Concentration of GM-CSF in the LPS serum obtained by $i.p.$ injection was 50.1 pg mL^{-1}. Furthermore, neither SpHW nor Phyc showed synergistic effect on bone marrow cells colony formation of the LPS serum, that is, the amount of colony formation by LPS serum with SpHW or Phyc was almost the same as that by the serum alone (data not shown).

Colony- and cluster-formation in the bone marrow cells with culture supernatants of the spleen (SP), Peyer's patch (PP), and peritoneal-exudated (PE) cells from the mice, which ingested the *Supirulina* extracts, SpHW, Phyc, and SpCW, for 5 weeks, were then measured in *in vivo* study. Culture supernatants of each lymphoid-organ, SP, PP, and PE cells from the groups were prepared under stimulation with or without phycocyanin. Colony formation by the culture supernatant of SP cells from the mice of SpHW group, as well as by that of PE cells from Phyc. or SpCW group, under stimulation with phycocyanin, was significantly higher than that by each culture supernatant of cells from control group (Figure 5A). A similar tendency of enhanced colony formation was observed especially in the culture supernatant of SP cells from SpHW group without phycocyanin, although the level of colony formation made by the culture supernatant without phycocyanin was smaller than that by culture supernatant with phycocyanin (Figure 5B).

While ratios of lymphocytes, neutrophils, and monocytes in the peripheral blood of control group were in the normal range, ratios of neutrophils in the SpHW group and lymphocytes in the SpCW were significantly higher than in controls. Ratio of neutrophils in SpCW group, however, was reduced (Figure 6A). A significant increase in ratio of lymphocytes was also observed in bone marrow cells in Phyc. group, although the number of cells was small (Figure 6B). In addition, increased ratio of reticulocytes was observed in the bone marrow of the mice fed with SpHW.

Discussion

Immunomodulation properties of *Spirulina* have been widely studied in chickens, prawns and fish, other animals, and humans. Generally, *Spirulina* and its extracts, such as hot-water extracts and phycocyanin, tended to enhance immune functions including mucosal or innate immunity through macrophage (Liu et al., 2000; Belay, 2002; Nemoto-Kawamura et al., 2004) and secretions of the related cytokines (Hirahashi et al., 2002). Recently, Mao et al. (2000) demonstrated that *Spirulina*

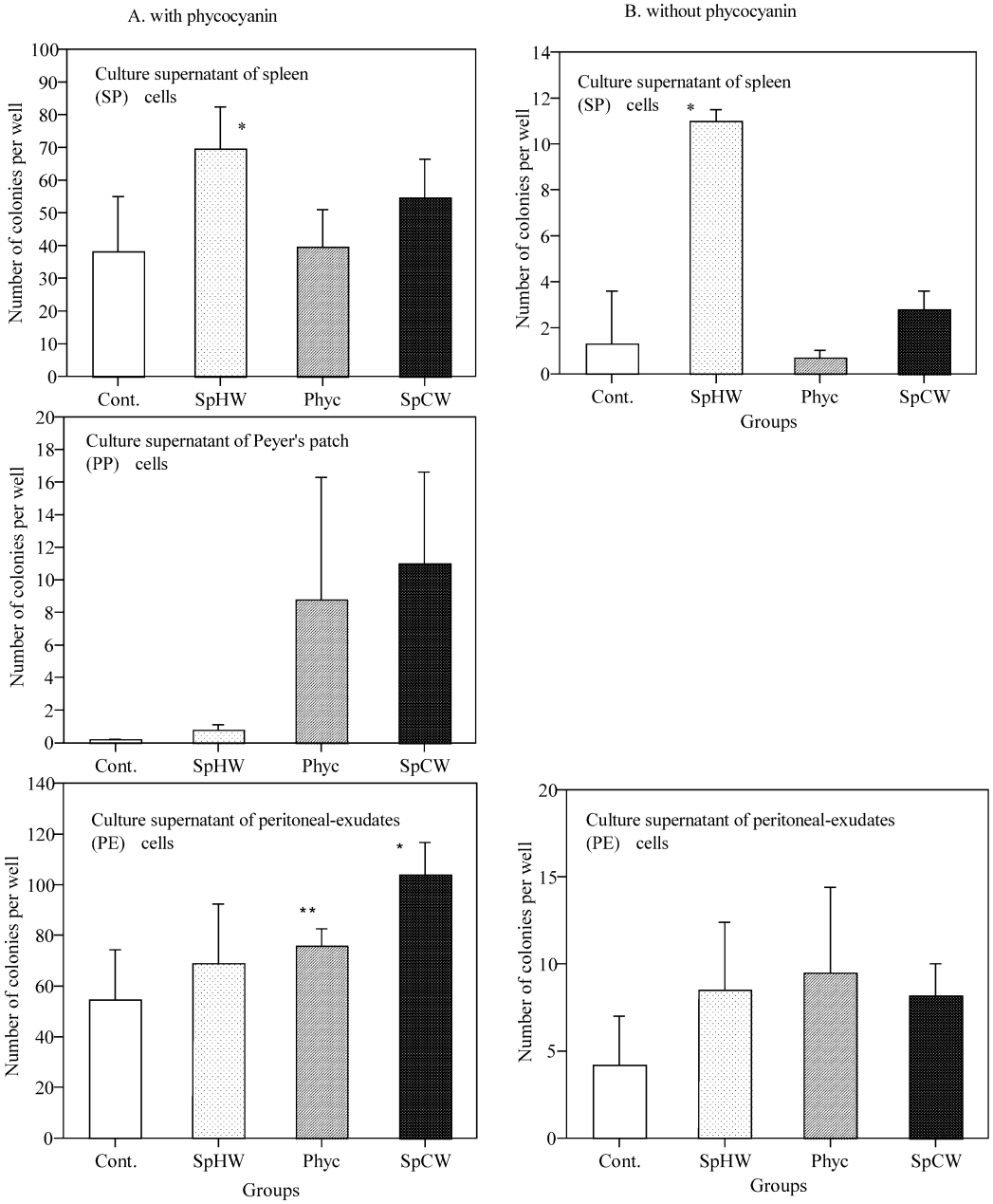

Figure 5. Bone marrow-cell colony formation in soft agar assay with the culture supernatants of each lymphoid organ, SP, PP, and PE cells from the mice fed with *Spirulina* extracts for 5 weeks. Culture supernatants of the cells from each lymphoid organ were prepared under stimulation with (A) or without (B) phycocyanin (values are mean ± SD, n = 3).

stimulated the secretion of IL-1ß and IFN-γ in humans and suggested that *Spirulina* helps balance the production of Th1 and Th2 cytokine stimulation.

The present study investigated the effects of *Spirulina* and its extracts on proliferation of bone marrow cells and induction of colony-forming activity to evaluate whether *Spirulina* has potentials to enhance or sustain immune functions as a consequence of promoting proliferation or differentiation of immune competent-cells. *Spirulina* extracts, SpHW, Phyc, and SpCW, as well as culture supernatants of the spleen cells stimulated with the extracts, enhanced proliferation of bone-marrow cells. The culture supernatants of the spleen cells, especially those stimulated with Phyc

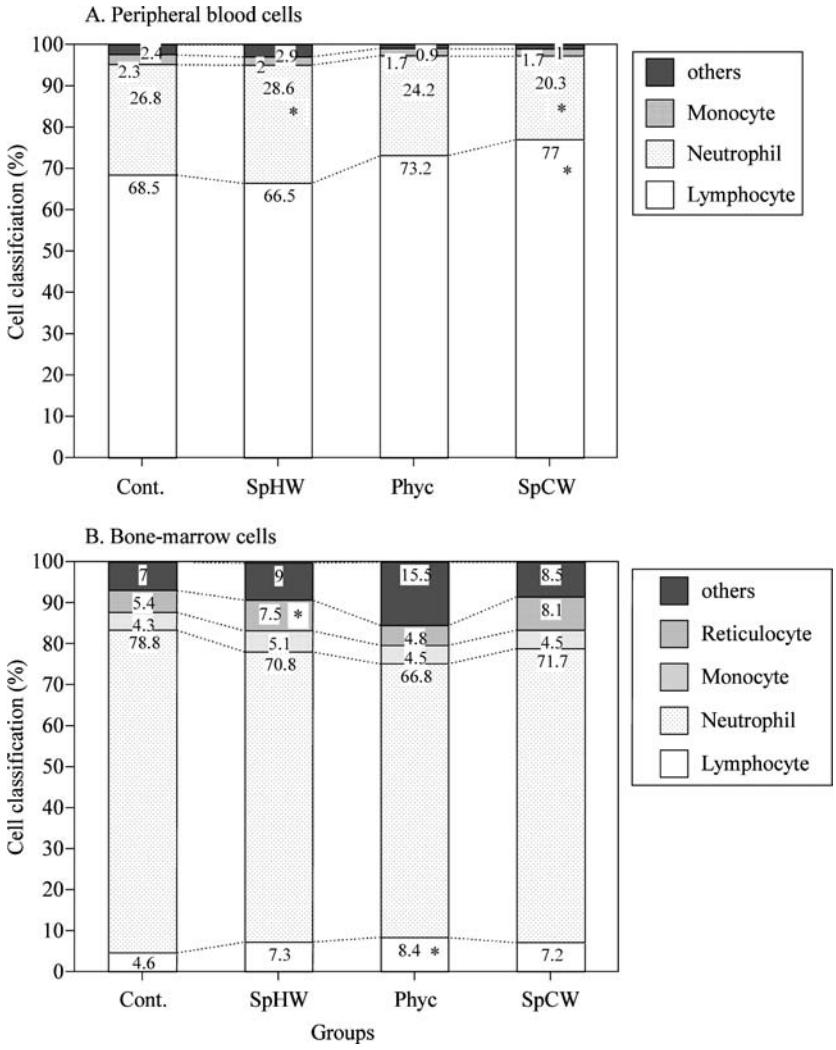

Figure 6. Classification of peripheral blood (A) and bone marrow (B) cells of the mice fed with *Spirulina* extracts for 5 weeks (values are mean, n = 6)

or SpCW, increased colony formation of bone marrow cells. Furthermore, high amounts of GM-CSF or IL-3 as a colony-forming activity were detected in the culture supernatants of the spleen cells or peritoneal-exudates cells stimulated with the *Spirulina* extracts, especially those with SpCW. In addition to the *in vitro* study, colony-forming activities in the sera of the mice fed with the *Spirulina* extracts, especially Phyc, for 5 consecutive days as well as intraperitoneally injected with the *Spirulina* extracts were also significantly increased.

It is known that multi-potent colony-stimulating factors such as G- and GM-CSF and IL-3, which are produced by a variety of cells including monocytes and lymphocytes can support proliferation of immature hematopoietic cells (Ihle, 1992). Valtieri et al. (1987) reported that in *in vitro* system using the IL-3-dependent granulocytic lineage 32D clone 3 (Cl3) cells derived from normal murine bone marrow, G-CSF stimulated terminal differentiation of the cells into neutrophilic granulocytes. Further, non-dialyzable extracts of some vegetables such as spinach induced the differentiation of other myeloid leukemia and promyelocytic cell lines U937 and HL-60 cells, respectively (Kobori and Shinohara, 1993; Kobori et al., 1995). Shinohara et al. (1988) reported that both phycocyanin and allophycocyanin from *Synechococcus elongatus*, a thermophilic blue-green alga, promoted the growth of a human myeloid cell line, RPMI 8226 cells. Recently, Liu et al. (2000) reported that phycocyanin

from *Spirulina platensis* inhibited growth of human leukemia K562 cells in a dose-dependent manner, arresting them at the G1 phase with increased level of *c*-myc expression, suggesting that phycocyanin may enhance differentiation of the leukemia cells. From these findings, it is suggested that *Spirulina*, including its components such as phycocyanin can affect enhancing proliferation or differentiation of immune competent-cells including bone marrow cell, which may cause normally sustaining or enhancing immune functions.

Colony-forming activity was also significantly induced in the blood, spleen, and Peyer's patch cells in the mice which ingested *Spirulina* extracts, SpHW, Phyc or SpCW, orally for 5 weeks in *in vivo* study although neither significant amount of GM-CSF nor IL-3 was detected in the blood (data not shown). Colony-stimulating activity other than IL-3 or GM-CSF, for example arginase and G-CSF, in the serum may contribute to the cell differentiation, although this is still not clear. Ratios of neutrophils and lymphocytes of the mice fed with *Spirulina* extracts, SpHW and SpCW, in fact, were consequently increased in the peripheral blood. In addition, ratios of reticulocytes by SpHW and lymphocytes by Phyc were increased in the bone marrow of the mice. Zhang et al. (1994) found that c-phycocyanin and polysaccharide isolated from *Spirulina* increased leukocyte and bone marrow nucleated cell counts as well as colony formation of colony forming unit-granulocyte and macrophage (CFU-GM) in the gamma-ray irradiated mice, and also found that c-phycocyanin possessed high erythropoietin activity. These support our present results.

Much evidence for clinical applications of hematopoietic growth factors such as GM-CSF and IL-3 to bone marrow failure patients has been accumulated until today. These approved cytokines as well as erythropoietin are effectively used to decrease cytopenias associated with high-dose chemotherapy, bone marrow transplantation, and leukemia patients (Vose and Armitage, 1995), and a phase I study of aerosolized GM-CSF recently demonstrated tolerance and possible efficacy in patients with malignant metastases to the lungs, possibly through upregulation of antigen-specific cytotoxic T-cells (Rao et al., 2003). Human immunodeficiency virus (HIV) infection commonly associates with impaired hematopoiesis, such as anemia, leukemia, and thrombocytopenia, in addition to bone marrow hyperplasia with dysmorphic changes. Many chemotherapeutics for patients with HIV-related diseases generally manifest myelosuppressive or marrow-suppressive nature. On the contrary to that, GM-CFS and granulocyte colony-stimulating factor (G-CSF) are known to increase neutrophil counts in such patients (Groopman & Feder, 1992; Scadden, 1997).

It is known that various food compounds and the metabolites involving phycocyanin in *Spirulina* can influence the processes in cellular differentiation, apoptosis, and proliferative potential, and there is considerable evidence that vitamins and micronutrients are able to regulate gene expression of cancer cells, resulting in influence on the carcinogenic process (Sacha et al., 2005). All-*trans*-retinoic acid and vitamin D3 are known as one of the physiologic agents which can modulate the proliferation and differentiation of hematopoietic cells (Collins, 2002) in addition to contributing to calcium homeostasis (Yoshida et al., 1992). The vitamin plus interferon-γ treatment and enrichment with polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid also significantly enhanced the expression of monocytic surface antigens CD11b and CD14 on human premonocytic U937 cells (Obermeier et al., 1995), or enhanced immunoregulatory effects. From these reports, in addition to our present results, it can be suggested that *Spirulina* is useful in providing complementary nutrients for modulating or maintaining the immune system and that is also may have potential therapeutic benefits for improvement of weakened immune functions caused by, for example, the use of anti-cancer and anti-infectious drugs or HIV-related diseases. These possibilities are under investigation in our laboratory.

References

Belay A (1997) Mass culture of *Spirulina* outdoor—The Earthrise Farm experience In: Vonshak A (ed.) *Spirulina platensis* (*Arthrospira*): Physiology, Cell-Biology and Biotechnology. Taylor & Francis Ltd., UK. pp. 131–158.

Belay A (2002) The potential application of *Spirulina* (*Arthorspira*) as a nutritional and therapeutic supplement in health management. J. Am. Nutraceut. Ass. 5: 27–48.

Belay A, Ota Y, Miyakawa K, Shimamatsu H (1993) Current knowledge on potential health benefits of *Spirulina*. J. Appl. Phycol. 5: 235–241.

Ciferri O (1983) *Spirulina*, the edible microorganisms. Microbiol. Res. 47: 551–578.

Collins SJ (2002) The role of retinoids and retinoic acid receptors in normal hematopoiesis. Leukemia 16: 1905–1986.

Groopman JE, Feder D (1992) Hematopoietic growth factors in AIDS. Seminars in Oncology 19: 408–414.

Hayashi O, Hirahashi T, Katoh T, Miyajjima H, Hirano T, Okuwaki Y (1998) Class specific influence of dietary *Spirulina platensis* on antibody production in mice. J. Nutr. Sci. Vitaminol. (Tokyo). 44: 841–851.

Hayashi O, Katoh T, Okuwaki Y (1994) Enhancement of antibody production in mice by dietary *Spirulina platensis*. J. Nutr. Sci. Vitaminol. (Tokyo). 40: 431–441.

Hirahashi T, Matsumoto M, Hazeki K, Saeki Y, Ui M, Seya T (2002) Activation of the human innate immune system by *Spirulina*: augmentaion of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. Int. J. Immunopharmacol. 2: 423–734.

Hudson L, Hay FC (1976) Practical Immunology. Blackwell Scientific, London. 298 pp.

Ihle JN (1992) Interleukin-3 and hematopoiesis. Chem. Immunol. 51: 65–106.

Kay RA (1991) Microalgae as food and supplement. Crit. Rev. Food Sci. Nutr. 30: 555–573.

Kobori M, Miyama Y, Tsushida T, Shinmoto H, Shinohara K (1995) Effect of non-dialyzable extracts of vegetables on the differentiation of U-937 human myeloid leukemia cell line. Nippon Shokuhin Kagaku Kogaku Kaishi 42: 61–68.

Kobori M, Shinohara K (1993) Effects of spinach extract on the differentiation of the human promyelocytic cell line, HL-60. Biosci. Biotechnol. Biochem. 57: 1951–1952.

Liu Y, Xu L, Cheng N, Lin L, Zhang C (2000) Inhibitory effect of phycocyanin from *Spirulina platensis* on the growth of human leukemia K562 cells. J. Appl. Phycol. 12: 125–130.

Mao TK, Van De Water J, Gershwin ME (2000) Effect of *Spirulina* on the secretion of cytokines from peripheral blood mononuclear cells. J. Med. Food 3: 135–140.

Metcalf D, Foster R (1967) Bone marrow colony-stimulation activity of serum from mice with viral-induced leukemia. J. Natl. Cancer Inst. 39: 1235–1245.

Moore G, Gerener R, Franklin H (1967) Culture of normal human leukocytes. JAMA 199: 519–524.

Nemoto-Kawamura C, Hirahashi T, Nagai T, Yamada H, Katoh T, Hayashi O (2004) Phycocyanin enhances secretory IgA antibody responses and suppresses allergic IgE antibody response in mice immunized with antigen-entrapped biodegradable microparticles. J. Nutr. Sci. Vitaminol. (Tokyo). 50: 129–136.

Obermeier H, Hrboticky N, Sellmayer A (1995) Differential effects of polyunsaturated fatty acids on cell growth and differentiation of premonocytic U937 cells. Biochim. Biophys. Acta 1266: 179–185.

Page B, Page M, Noël C (1993) A new fluorometric assay for cytotoxicity measurements in vitro. Int. J. Oncol. 3: 473–476.

Rao RD, Anderson PM, Arndt CA, Wettstein PJ, Markovic SN (2003) Aerosolized granulocyte macrophage colony-stimulating factor (GM-CSF) therapy in metastatic cancer. Am. J. Clin. Oncol. 26: 493–498.

Sacha T, Zawada M, Hartwich J, Lach Z, Polus A, Szostek M, Zdzitowska E, Libura M, Bodzioch M, Dembinska-Kiec A, Skotnicki A, Goralczyk R, Wertz K, Riss G, Moele C, Langmann T, Schmitz G (2005) The effect of beta-carotene and its derivatives on cytotoxicity, differentiation, proliferative potential and apoptosis on the three human acute leukemia cell lines: U-937, HL-60 and TF-1. Biochim. Biophys. Acta. 1740: 206–214.

Scadden DT (1997) Cytokine use in the management of HIV disease. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 16(Suppl. 1): S23–S29.

Shinohara K, Okura Y, Koyano T, Murakami H, Omura H (1988) Algal phycocyanins promote growth of human cells in culture. In Vitro Cell. Dev. Biol. 2: 1057–1060.

Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D, Rovera G (1987) Cytokine-dependent granulocytic differentiation. Regulaion of proliferative and differentiative responses in a murine progenitor cell line. J. Immunol. 138: 3829–3835.

Vose JM, Armitage JO (1995) Clinical applications of hematopoietic growth factors. J. Clin. Oncol. 13: 1023–1035.

Yoshida M, Tanaka Y, Eguchi T, Ikekawa N, Nagahiro S (1992) Effect of hexafluoro-1,25-dihydoxy vitamin D3 and sodium butyrate combination on differentiation and proliferation of HL-60 leukemia cells. Anticancer Res. 12: 1947–1952.

Zhang C-W (1994) Effect of polysaccharide and phycocyanin from *Spirulina* on peripheral blood and hematopoietic system of bone marrow in mice. Proceeding of 2nd Asia Pacific Conference on Algal Biotechnology, China: 58.