Class Specific Influence of Dietary *Spirulina platensis* on Antibody Production in Mice

Osamu Hayashi,* Tomohiro Hiraishi,1 Toshimitsu Katoh,1 Hiroaki Miyajima,2 Takao Hirano2 and Yoshiyuki Okuwaki

Department of Health and Nutrition, Kagawa Nutrition University, Sakado 350–0288, Japan.
1 Biochemical Division, Dainippon Ink & Chemicals Inc., Ichihara, Chiba 290–8585 Japan
2 Department of Immunology, Juntendo University School of Medicine, Hongo, Tokyo 113–8421, Japan

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Summary In the present study, we investigated antibody productions of IgA and other classes, such as IgE and IgG1, in mice as possible evidence of the protective effects of *Spirulina* toward food allergy and microbial infection. An increase of IgE antibody level in the serum was observed in the mice that were orally immunized with crude shrimp extract as an antigen (Ag group). The antibody level, however, was not further enhanced by treatment with *Spirulina* extract (SpHW). IgG1 antibody, on the other hand, which was increased by antigen administration, was further enhanced by *Spirulina* extract. It was noted that the IgA antibody level in the intestinal contents was significantly enhanced by treatment with *Spirulina* extract concurrently ingested with shrimp antigen, in comparison with that of the Ag group treated with shrimp antigen alone. An enhancement of IgA antibody production by *Spirulina* extract was also observed in culture supernatant of lymphoid cells, especially in the spleen and mesenteric lymph node from mice treated with *Spirulina* extract for 4 weeks before antigen stimulation. These results suggest that *Spirulina* may at least neither induce nor enhance allergic reaction such as food allergy dependent on an IgE antibody, and that when ingested both concurrently with antigen and before antigen stimulation, it may significantly enhance the IgA antibody level to protect against allergic reaction.

**Key Words** *Spirulina platensis*, IgA, IgE, IgG1, mucosal immunity

*Spirulina platensis*, which is a helicoidal filamentous blue-green alga or cyanobacterium, has a history of being used as food for more than a thousand years (1, 2). It has been commercially produced for almost 20 years as a human...
food supplement because of the advantages of mass cultivation and easy harvest of the micro alga (3). Nutritional studies have demonstrated that it contains high-quality protein and other nutritional components such as vitamins; minerals; essential fatty acids, including \( \gamma \)-linolenic acid; and \( \beta \)-carotene (3–5). Recently, more attention has been given to the study of the therapeutic effects of *Spirulina*. Besides reports concerning its effectiveness in reducing hyperlipidemia, diabetes, and high blood pressure in man and animals, antitumor and antiviral effects against *Herpes simplex* have also been reported (6). We reported in the previous paper that immune response to a thymus-dependent antigen in mice fed with *Spirulina platensis*-added feeds was significantly enhanced (7).

In the present study, we focused on antibody productions of IgA and other classes such as IgE and IgG1 in mice treated with *Spirulina* extract that were concurrently or protectively ingested with orally administered antigen.

**MATERIALS AND METHODS**

*Animals.* Female C3H/HeJ Jcl mice aged 5 weeks (Clea Japan, Japan) were used. The mice were housed 5 per polycarbonate cage (W21 × D33 × H13 cm) with wood-flake bedding. They were kept in a constant temperature (25 ± 1°C) and a relative humidity (60 ± 5%) with a 12 h light period from 08:00 to 20:00 and were fed normal laboratory chow, CE-2 (Clea Japan). Water and diluted *Spirulina*-extract solution, which were sterilized by filtration through a 0.45-µm pore filter, were supplied by using an aseptic nursing pack (AN pack) with an exclusive nozzle (Musashi, Japan) ad libitum during experiments.

*Preparation of Spirulina extract (SpHW) and its diluted solutions, SPC and SPD.* *Spirulina platensis*, harvested from the culture in outdoor open tanks and spray-dried after washing, was obtained from Dainippon Ink & Chemicals (Tokyo, Japan). One kilogram of dried *Spirulina* was suspended in 10 L of water and extracted at 120°C, 2 atm, for 1 h. After cooling to room temperature and adjusting pH to 4.0 with citric acid, the suspension was centrifuged with Celite to remove cell debris. Supernatant was concentrated to 1.5–1.6 L after another centrifugation with Celite. Carbohydrate and protein contents in the concentrate of *Spirulina* extract (SpHW) were 14.5 mg/mL as glucose and 0.78 mg/mL as bovine serum albumin (BSA), respectively, measured by the phenol-sulfuric acid method (8) and Bio-Rad Protein assay based on the Bradford method (9). SpHW was frozen at -20°C; diluted to 15- and 60-fold and designated SPC and SPD, respectively; when used, it was sterilized by filtration as described above.

*Preparation of crude shrimp as an immunized antigen.* Crude shrimp extract for immunization was prepared as described by Waring et al (10) with minor modifications. Briefly, 1 kg of raw white shrimp (product of Indonesia, Toho Suisan, Japan) was shelled, and the meat was homogenized in 1,000 mL of phosphate-buffered saline (PBS), pH 7.2, in a Waring blender. The homogenate was stirred overnight at 4°C and centrifuged at 27,000 × g, and the resulting
supernatant was concentrated by ultrafiltration with a DIAFLO membrane filter, YM-3 (AMICON, MA). The concentrated supernatant was sterilized by filtration after recentrifugation at 78,000 × g and stored at −20°C. The dry weight of 1 mL of the crude shrimp extract was 30 mg, and protein content was 16.3 mg/mL by Bio-Rad protein assay, as BSA.

Experiment A. Measurement of antibody levels in the blood and intestinal contents of mice treated with Spirulina extract concurrently ingested with shrimp antigen—Schedule of Spirulina ingestion and immunization. Twenty-four mice used in the experiment were given sterilized water for the first week and divided into 4 groups, shown in Fig. 1A. Two groups, Cont. and Ag, were given sterilized water, and the other groups, Ag-SPC and Ag-SPD, were given diluted Spirulina extract solutions, SPC and SPD, respectively, in aseptic AN packs for 5 weeks during the experiment. Three groups of the mice—Ag, Ag-SPC, and Ag-SPD—were immunized intraperitoneally with 400 μg crude shrimp extract and inactivated Bordetella pertussis adjuvant (1 × 10¹⁰ cells; Wako Pure Chemical Industries, Japan) per 0.5 mL PBS per mouse as primary immunization. Following the primary immunization, 1 mg of crude shrimp per 0.2 mL sterilized PBS per mouse was orally given to each mouse twice a week via an animal feeding catheter inserted gently into the stomach. The administration of shrimp antigen was done concurrently with the ingestion of Spirulina extract. For the control group (Cont.), sterilized PBS was intraperitoneally injected and orally administered.

Five weeks after the primary immunization, all groups of the mice were exsanguinated from the femoral artery under ether anesthesia. Each serum sample was obtained by centrifugation at 1,500 × g. The intestinal contents of each mouse were collected from whole gut lumen, from the duodenum to the ileum, by washing with PBS. The intestinal contents suspended in PBS were centrifuged at 2,150 × g, filtered through 0.45 μm-pore disk filter, and designated as IC. These samples were kept at −40°C until assay.

Experiment B. Measurement of antibody levels in culture supernatants of lymphoid cells from mice treated with Spirulina extract ingested before antigen stimulation—Schedule of Spirulina ingestion and immunization. Twenty mice were divided into 4 groups, and sterilized water was given to 2, Cont. and Ag. Sixty-fold diluted sterilized Spirulina extract (SPD) was protectively given to the SPD-Ag group for 4 weeks before and one week after primary immunization (Fig. 1B), and also to the SPD group. An oral administration of shrimp antigen following the primary immunization was done once as described above.

One week after primary immunization, the spleens and mesenteric lymph nodes were aseptically removed from 2 or 3 mice of each group and collected together and gently homogenized in Hank's balanced salt solution (HBSS, GIBCO Lab., NY). The resulting single cells of each lymphoid organ were adjusted to 2 × 10⁸ cells/mL of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, GIBCO Lab.) and 0.05 mM 2-mercaptoethanol. The Peyer's patches removed from 5 mice of each group were collected together and treated with Dispase (Becton
A. Treatment of Spirulina extract concurrently ingested with shrimp antigen in Experiment A.

- **Cont.**
  - n = 6
  - **Sterilized H₂O** △ (0w) ▽ (5w) for 1 week PBS (0.2 mL/mouse) po, twice a week
- **Ag**
  - n = 6
  - **Sterilized H₂O** ▲ (0w) ▽ (5w) for 1 week Crude shrimp (1.0 mg/mouse) po, twice a week
- **Ag-SPC**
  - n = 6
  - **Sterilized H₂O** ▲ (0w) ▽ (5w) for 1 week 15-fold diluted Sp. extract (SPC)
- **Ag-SPD**
  - n = 6
  - **Sterilized H₂O** ▲ (0w) ▽ (5w) for 1 week 60-fold diluted Sp. extract (SPD)

B. Treatment of Spirulina extract ingested prior to antigen stimulation in Experiment B.

- **Cont.**
  - n = 5
  - **Sterilized H₂O** △ (0w) ▽ (1w) for 4 weeks PBS (0.2 mL/mouse) po, once
- **Ag**
  - n = 5
  - **Sterilized H₂O** ▲ (0w) ▽ (1w) for 4 weeks Crude shrimp (1.0 mg/mouse) po, once
- **SPD**
  - n = 5
  - **SPD** △ (0w) ▽ (1w) for 4 weeks PBS (0.2 mL/mouse) po, once
- **SPD-Ag**
  - n = 5
  - **SPD** ▲ (0w) ▽ (1w) for 4 weeks Crude shrimp (1.0 mg/mouse) po, once

▲; ip injection as primary immunization by 400 μg of shrimp with inactivated *Bordetella pertussis* (1x10⁸ cells/0.5mL)
△; PBS 0.5 mL, ip injection
▽; collected blood samples and intestinal contents in A.
▽; collected spleen, mesenteric lymph node, and Peyer’s patch in B.

Fig. 1. The schedules of Spirulina extract ingestion and immunization to C3H/HeJ Jcl mice in Experiments A and B. Five or 6 female mice (4 weeks old) per group were used in the experiments.
Dickinson Labware, MA) at 37°C water bath with shaking for 60 min. The dissociated cells, obtained after being flashed several times, were adjusted to $2 \times 10^6$ cells/mL of the medium. The spleen, mesenteric lymph node, and Peyer’s patch cells were cultured for 4 d on 5 wells of 24-well plates in each group in a humidified atmosphere of 5% CO$_2$ and 95% air at 37°C. Culture supernatant in each well was collected after filtration through a 0.45 μm-pore disk filter to remove cell debris and kept at −40°C until measurement of the antibody levels.

Measurement of mouse IgE, IgG1, and IgA antibodies. In the enzyme-linked immunosorbent assay (ELISA) method for IgE antibody, rat monoclonal antibody 6HD5 and Biotin-labeled antibody HMK-12, which were specific for murine IgE, were used as the first and second antibodies, respectively, and SPE7 IgE antibody was used as the standard (11). Fifty microliters of 10-fold diluted serum sample or nondiluted culture supernatant were added to each well of a 96-well plate (Immulon II, Dynatec Laboratories, VA) and assayed by the method previously described (11). All samples were assayed in duplicate.

For a measurement of the IgA antibody, rabbit antimouse IgA (ZYMED Laboratories, CA; code No. 61-6700), peroxidase-conjugated rabbit antimouse IgA (HRP-rabbit antimouse IgA, ZYMED Laboratories; code No. 61-6720), and purified mouse myeloma IgA (ZYMED Laboratories; code No. 02-6500) were used. For a measurement of the IgG1 antibody, rabbit antimouse IgG and HRP-rabbit antimouse IgG1 (ZYMED Laboratories; code Nos. 61-6000 and 61-0120, respectively) and purified mouse myeloma IgG1 (ZYMED Laboratories; code No. 02-6100) were used. Fifty microliters of 7,500-fold diluted serum or 50,000-fold diluted intestinal contents (IC) and 2-fold diluted culture supernatant were added to each well of the plate and assayed by the ELISA method (12). All samples were assayed in duplicate.

For a measurement of antigen-specific IgE, IgG1, and IgA antibodies, the serum sample and IC were diluted 10-fold with PBS.

Passive cutaneous anaphylaxis (PCA) reaction. PCA reaction was carried out according to Ovary et al (13). Briefly, 0.2 mL each of every 2-fold (from 10- to 80-fold) diluted serum samples of Ag, Ag-SPC, and Ag-SPD groups in Experiment A were injected intradermally on the hair-clipped dorsal surface of 3 Crj:CD(SD) rats (Charles River Japan, Yokohama). Following a 2-h sensitization period, the PCA reactions were elicited by challenging the rats intravenously with a mixture of 2.5 mg crude shrimp and 5 mg Evans blue in 1 mL of PBS. After 30 min, the rats were asphyxiated with ether and skinned to measure the diameters of the blue spots on the inner surface. The spots whose two-directional diameters were larger than 10 mm were classified as positive reaction, and the maximum dilution of the serum showing positive reaction was recorded as PCA titer of the serum.

Statistical analysis. The statistical significance of the difference between the experimental and control groups was determined by Student’s $t$ test or analysis of variance (ANOVA) by using StatView v.4.5 (Abacus Concepts, CA).
RESULTS

Body weight gain of mice and Spirulina intake

Each group of C3H/HeJ Jcl mice treated with Spirulina extract gained body weight normally during experiments. The average body weights of the groups, Cont., Ag, Ag-SPC, and Ag-SPD, were 20.1±1.0 g, 20.2±1.1 g, 18.8±0.8 g, and 19.2±2.1 g, respectively, at the last period of the experiment. Among these groups, Ag-SPC showed significantly low body weight (p<0.05) compared with Cont. The average volumes of diluted Spirulina extract ingested during the experiment (for 5 weeks) were 3.0 mL of SPC and 3.4 mL of SPD per mouse per day in the Ag-SPC and Ag-SPD groups, respectively.

Antibody levels in blood samples and intestinal contents of mice treated with Spirulina extract concurrently ingested with antigen stimulation in Experiment A

As shown in Fig. 2, the total IgA antibody in the intestinal contents and the IgG1 in the serum of the Ag-SPD group were significantly enhanced in comparison with those of the Cont. and Ag groups. Antigen-specific IgA and IgG1 antibody levels similarly tended to be increased by Spirulina ingestion in the Ag-SPC or Ag-SPD group, though significant difference was not observed. Antigen-specific IgE antibody levels in the Ag, Ag-SPC, and Ag-SPD groups treated with shrimp antigen were significantly high in comparison with the Cont. group. The total and the antigen-specific IgE antibody levels of Ag-SPC and Ag-SPD were almost the same as those of the Ag group (Fig. 2B). The differences of average PCA titers among the Ag, Ag-SPC, and Ag-SPD groups were not significant by ANOVA, though the total and antigen-specific IgE antibody titers in the sera showed significant correlation to PCA titers measured on the dorsal surface of Crj: CD rats. The correlation coefficients to total and antigen-specific IgE were 0.768 and 0.898, respectively (n=16, p<0.01).

IgA and IgG1 antibody levels in culture supernatants of lymphoid cells from mice treated with Spirulina extract ingested before antigen stimulation in Experiment B

As shown in Fig. 3, total IgA and IgG1 antibody productions in the culture supernatant of lymphoid cells from the mice of SPD-Ag, which was treated with Spirulina extracts followed by antigen stimulation, were greatly increased, especially in the spleen and mesenteric lymph node cells (p<0.01). The IgA level in the spleen and mesenteric lymph node cell culture of the SPD-Ag group was significantly increased in comparison with that of the Ag group treated with antigen stimulation but not with Spirulina extract. The increases of the total and antigen-specific IgG1 antibody levels in the spleen or mesenteric lymph node cell culture in SPD-Ag were not significant in comparison with those of the Ag group, except for a decrease of total IgG1 of the mesenteric lymph node cells. The IgA and IgG1 antibody production of the SPD group treated with Spirulina extract without antigen stimulation either was not affected or was slightly increased. The
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Fig. 2. IgA and other antibody productions in mice treated with Spirulina extract concurrently ingested with antigen stimulation. Three groups of mice—Ag, Ag-SPC, and Ag-SPD—in Experiment A were primarily immunized by crude shrimp antigen with B. pertussis as an adjuvant. Shrimp antigen was then orally administered to each mouse twice a week during the experiment with or without ingestion of Spirulina extract. The Cont. group was treated with PBS and sterilized water. Antibody levels in the serum and intestinal contents (IC) of each mouse were measured by ELISA. Values are means ± SD for six determinations of each group. In Student’s t-test: * $p<0.05$; ** $p<0.01$ compared with Cont; * $p<0.05$; ** $p<0.01$ compared with the Ag group.

IgE antibody level in the culture supernatant of the lymphoid cells of the groups was less than the detection limit of the ELISA method.

DISCUSSION

Several published and unpublished studies have shown significant therapeutic effects of Spirulina or its extracts on animals and on humans. For example, it has been shown to have beneficial effects on hyperlipidemia, hypertension, diabetes, and atopic dermatitis and to exert antitumor effects (5). Besides the extensive use of Spirulina as a food supplement for humans, it has recently been used increasingly as an animal feed supplement. Some recent studies have shown that feeding Spirulina...
Fig. 3. IgA and IgG1 antibody productions in the culture supernatant of lymphoid cells from mice treated with *Spirulina* extract before antigen stimulation in Experiment B. Diluted *Spirulina* extract (SPD) was protectively given to the SPD-Ag and the SPD groups for 4 weeks followed by primary immunization and oral administration of shrimp antigen as described in Fig. 2. The Cont. and Ag groups were treated with sterilized water. One week after primary immunization, the spleens, mesenteric lymph nodes, and Peyer’s patch were aseptically removed. The cell suspensions of each lymphoid organ were adjusted to 2 × 10^6 cells/mL of RPMI-1640 medium supplemented with 10% fetal calf serum and 0.05 mM 2-mercaptoethanol and cultured for 4 d on 5 wells of 24-well plates in each group. The antibody levels in culture supernatant were measured by ELISA. The values are means ± SD for five determinations of each group. In Student’s t-test: * p < 0.05; ** p < 0.01 compared with the Cont. group; ++ p < 0.01 compared with the Ag group.

to fish and poultry results in increased disease resistance and in improved survival and growth rates, which may be attributed to an improvement of immune functions (14). Some reports have cited the suppression of delayed hypersensitivity by toluene-2,4-diisocyanate in mice fed *Spirulina* in chow (15) and the antitumor effect of *Spirulina* in hamsters through stimulation of the immune response, involving T-cell activation (16). But immunological studies of *Spirulina* have been scarce.

We previously reported that the immune response of mice fed with whole cells of *Spirulina platensis* was significantly increased, especially the numbers of splenic IgM-producing cells in the primary response, partly through the enhancement of
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macrophage functions such as phagocytosis and interleukin 1 (IL-1) induction (7). Moreover, it has been reported that intraperitoneally injected polysaccharides of a hot-water extract of Spirulina increased the percentage of peritoneal phagocytic cells besides increasing the hemolysin contents in the blood of mice (17). Recently, a sulfated polysaccharide named calcium spirulan, isolated from a hot-water extract of Spirulina, was found to show an antiviral effect, that is, to inhibit the replication of several enveloped viruses, including HIV-1 and Herpes simplex (6). In the present study, we investigated antibody productions of IgA and other classes in mice, such as IgE and IgG1, as possible sources of evidence of the protective effects of a hot-water extract of Spirulina toward food allergy and microbial infection. Orally administered crude shrimp extract was used as a stimulating antigen in the study because a significant induction of IgE antibody response following serial oral injection of the antigen in C3H/HeJ mice has been reported elsewhere (10, 18). In the preliminary study, an ingestion of Spirulina extract alone did not increase the basal level of IgE antibody up to 5 weeks, whereas the IgE antibody level increased within 3 or 4 weeks following serial shrimp antigen stimulation (data not shown). As shown in Fig. 2B, the antigen-specific IgE antibody was significantly increased in mice of the Ag group, which was orally immunized with shrimp antigen without Spirulina extract. The increase of the IgE antibody level by antigen stimulation observed in the Ag group, however, was not further enhanced by Spirulina concurrently ingested with antigen, as seen in the Ag-SPC and Ag-SPD groups. These results suggest that Spirulina appears neither to induce nor to enhance allergic reaction dependent on an IgE antibody such as food allergy. On the other hand, the IgG1 antibody, which was increased by orally immunized antigen (Fig. 2C), was further enhanced by the treatment of Spirulina extract, as observed in the Ag-SPD group. We further investigated the effects of Spirulina extract on IgA antibody production in the intestine of mice. The basal level of antibody was not affected by orally immunized antigen alone. It was noted that IgA antibody levels of mice in the Ag-SPD group, which were treated with Spirulina extract concurrently ingested with antigen, were significantly higher than those of the Ag and Cont. groups (Fig. 2A).

The enhancement of IgA antibody production by Spirulina extract was further confirmed in Experiment B, as shown in Fig. 1. The IgA antibody production in the cultured cells of lymphoid organs from mice treated with Spirulina extract for 4 weeks before antigen stimulation was examined. The IgA antibody level in the culture supernatant of lymphoid cells, especially in the spleen and mesenteric lymph node cells of the SPD-Ag group, resulted in significant enhancement of the antibody level in comparison with the Cont. and Ag groups (Fig. 3A), whereas neither antigen stimulation alone nor the administration of Spirulina extract alone increased the IgA antibody level in any lymphoid organ. These results suggest that simultaneous treatment with antigen and Spirulina extract may enhance IgA production through a stimulation of the intestinal immune system.

Numerous studies in humans and animals have provided convincing evidence

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that protection against a variety of viral and bacterial mucosal pathogens can be obtained by oral or intranasal immunization (19). Secretory IgA antibodies, the predominant isotype in most secretory tissues or mucosal surfaces, exhibit various biological properties such as agglutination of microorganisms; neutralization of bacterial enzymes, toxins, and viruses; immune exclusion; and inhibition of antigen absorption. They also exhibit synergistical interaction with antibacterial substances such as lysozyme and lactoferrin (19). Cholera toxin, bacterial lipopolysaccharide or lipid A, muramyl dipeptide, and a synthetic or nonbacterial lipoidal amine, avidin [N,N-dioctadecyl N'-N'-bis(2-hydroxyethyl)propanediamine], are known as mucosal adjuvants and have been found to potentiate secretory immune response to stimulate the production of IgA antibody (20). It has also been known that orally ingested lactic acid bacteria—Bifidobacterium longum, B. breve, and Lactobacillus casei—increased mucosal IgA response to antigen in vitro or in vivo studies in animals and in humans (21, 22). The role of cytokines in orchestrating the mucosal immune response has been greatly investigated for the potential of therapeutic applications to improve mucosal responses and to control systemic autoimmunity (23). Further experimentation concerning the mechanisms of stimulating local immune response by Spirulina, such as in regard to cytokine production, is necessary and is under investigation, besides investigations of the enhancing effect of Spirulina on IgA production in humans.

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REFERENCES

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