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Inhibitory Effect of Mast Cell-Mediated Immediate-Type Allergic Reactions in Rats by Spirulina

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ABSTRACT. We investigated the effect of spirulina on mast cell-mediated immediate-type allergic reactions. Spirulina dose-dependently inhibited the systemic allergic reaction induced by compound 48/80 in rats. Spirulina inhibited compound 48/80-induced allergic reaction 100% with doses of 100–1000 μg/g body weight, i.p. Spirulina (10–1000 μg/g body weight, i.p.) also significantly inhibited local allergic reaction activated by anti-dinitrophenyl (DNP) IgE. When rats were pretreated with spirulina at a concentration ranging from 0.01 to 1000 μg/g body weight, i.p., the serum histamine levels were reduced in a dose-dependent manner. Spirulina (0.001 to 10 μg/mL) dose-dependently inhibited histamine release from rat peritoneal mast cells (RPMC) activated by compound 48/80 or anti-DNP IgE. The level of cyclic AMP in RPMC, when spirulina (10 μg/mL) was added, transiently and significantly increased about 70-fold at 10 sec compared with that of control cells. Moreover, spirulina (10 μg/mL) had a significant inhibitory effect on anti-DNP IgE-induced tumor necrosis factor-α production. These results indicate that spirulina inhibits mast cell-mediated immediate-type allergic reactions *in vivo* and *in vitro*. BIOCHEM PHARMACOL **55**;7:1071–1076, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. spirulina; allergic reactions; cAMP; tumor necrosis factor-α

As part of our continuing search for biologically active antiallergic agents from medicinal sources, spirulina (cyanobacteria) was analyzed. Spirulina is a source of many biochemicals. The mast cell has long been thought to play a crucial role in the development of many physiological changes during anaphylactic and allergic responses [1]. Among the preformed and newly synthesized inflammatory substances released upon degranulation of mast cells, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of type I allergic reactions [2]. Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells [3]. The most potent secretagogues include the synthetic compound 48/80 and polymers of basic amino acids [4]. This mixture of polymers is synthesized by condensing *N*-methyl-*p*-methoxyphenyl ethylamine with formaldehyde [5], and its hypotensive effect was shown by Paton [6] to be the result of histamine release. Compared with the natural process, a high concentration of compound 48/80 induces an almost 90% release of histamine from mast cells. Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic reaction [7]. The secretory response of mast cells can also be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen [8, 9]. It has been established that the anti-IgE antibody induces PCA§ as a typical model for the mast cell-mediated immediate hypersensitivity [10]. Although mast cells also store small amounts of cytokines in their granules [11], these cells dramatically increase their production of TNF-α, interleukin-6, and other cytokines within 30 min after their surface FcεRI receptors are cross-linked with specific antigen [12–15].

In the present study, we showed that spirulina inhibited both compound 48/80-induced systemic allergic reaction and anti-DNP IgE antibody-induced PCA reaction. We also investigated the influence of spirulina on compound 48/80-induced intracellular cAMP level and anti-DNP IgE-induced TNF-α production in RPMC.

MATERIALS AND METHODS

Materials

Spirulina (cyanobacteria), compound 48/80, anti-DNP IgE, DNP-HSA, and metrizamide were purchased from the Sigma Chemical Co. α-MEM was purchased from Flow Laboratories, and FBS was purchased from Gibco Laboratories. The original stock of Wistar rats was purchased from the Dae-Han Experimental Animal Center, and the animals were maintained at the College of Pharmacy,

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§ *Abbreviations:* PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; RPMC, rat peritoneal mast cells; TNF-α, tumor necrosis factor-α; HSA, human serum albumin; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; and cAMP, cyclic AMP.

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Wonkwang University. The animals were housed 5–10 per cage in a laminar airflow room maintained under a temperature of 22 ± 1° and relative humidity of 55 ± 10% throughout the study.

Systemic Allergic Reaction

Rats were given an i.p. injection of compound 48/80, 8 μg/g body weight. Spirulina was dissolved in saline and administered i.p. 1 hr before the injection of compound 48/80. Mortality was monitored for 1 hr after induction of anaphylactic reaction. After the mortality test, blood was obtained from the heart of each mouse.

PCA

An IgE-dependent cutaneous reaction was generated by sensitizing the skin with anti-DNP IgE followed 48 hr later with DNP-HSA. Rats (N = 4) were injected with anti-DNP IgE (0.5 μg/site, i.d.) into each of four dorsal skin sites that had been shaved 48 hr earlier. The sites were outlined with a water-insoluble red marker. Each rat received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:4) via the tail vein 48 hr later. Spirulina was administered i.p. 1 hr before the challenge. Thirty minutes after the challenge, the rats were killed, and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 mL of 1.0 N KOH and 9 mL of a mixture of acetone and phosphoric acid (5:13) based on the method of Katayama *et al.* [16]. The absorbent intensity of the extraction was measured at 620 nm using a spectrophotometer, and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of Serum and Histamine Determination

Blood was centrifuged at 400 *g* for 10 min. The serum was withdrawn, and the histamine content was measured by the *o*-phthalaldehyde spectrofluorometric procedure of Shore *et al.* [17]. The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer.

Preparation of RPMC

RPMC were isolated as previously described [18]. In brief, rats were anesthetized by ether and injected with 20 mL of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, KCl, NaH_2PO_4) containing 0.1% gelatin (Sigma), in the peritoneal cavity, and the abdomen was massaged gently for about 90 sec. The peritoneal cavity was opened carefully, and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150 *g* for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes, according to the method described by Yurt *et al.* [19]. In brief, peritoneal cells suspended in 1 mL Tyrode buffer B were layered on 2 mL of 22.5% (w/v) metrizamide (density, 1.120 g/mL, Sigma) and centrifuged at room temperature for 15 min at 400 *g*. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A (10 mM of HEPES, 130 mM of NaCl, 5 mM of KCl, 1.4 mM of $CaCl_2$, 1 mM of $MgCl_2$, 5.6 mM of glucose) containing 0.1% bovine serum albumin (Sigma). Mast cell preparations were about 95% pure, as assessed by toluidine blue staining. More than 97% of the cells were viable, as judged by trypan blue uptake.

Inhibition of Histamine Release

Purified RPMC were resuspended in Tyrode buffer A containing calcium for the treatment of compound 48/80. RPMC (2×10^5 cells/mL) were preincubated for 10 min at 37° before the addition of compound 48/80 (1 μg/mL). Spirulina was dissolved in distilled water and then filtered through a 0.45–μm filter. Aqueous-form concentrations of spirulina were determined in each experimental group and subtracted from the amount obtained with filtration. The cells were preincubated with the spirulina preparations, and then incubated (10 min) with compound 48/80. RPMC (2×10^5 cells/mL) were sensitized with 10 μg/mL of anti-DNP IgE for 2 hr and preincubated with spirulina at 37° for 10 min prior to the challenge with 1 μg/mL of DNP-HSA. The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400 *g* for 5 min at 4°. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 *g* for 5 min at 4°.

Assay of Histamine Release

The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = \text{Histamine release without spirulina} - \text{Histamine release with spirulina} \times 100/\text{Histamine release without spirulina}$$

Measurement of cAMP Level

The cAMP level was measured according to the method of Peachell *et al.* [20]. In brief, RPMC were resuspended in prewarmed (37°) Tyrode buffer A. Typically, an aliquot of cells (5×10^5 cells) was added to an equivalent volume (50 μL) of prewarmed buffer containing the drug (10 μg/mL) in an Eppendorf tube. Spirulina significantly inhibited histamine release from RPMC at a concentration of 10 μg/mL. Therefore, we selected a 10 μg/mL concentration. The reaction was allowed to proceed for discrete time intervals, terminated by the addition of ice-cold acidified ethanol

(0.9 mL of 86% ethanol/1 M of HCl, 99:1) with brief vigorous vortexing and then snap frozen in liquid nitrogen. The sample was later thawed and vortexed; then the debris was sedimented in a centrifuge (400 *g* at 4° for 5 min), and an aliquot (0.9 mL) of the supernatant was removed and evaporated to dryness under a reduced pressure. The dried sample was reconstituted in assay buffer (150–200 μL) and stored frozen. The cAMP level was determined by enzyme immunoassay, using a commercial kit (Amersham International plc).

Assay of TNF-α Production

RPMC were resuspended in Tyrode buffer A. The cells were sensitized with anti-DNP IgE (1 μg/mL) and incubated for 6 hr in the absence or presence of spirulina before the challenge with DNP-HSA (0.1 μg/mL). TNF-α secretion was measured by a modified ELISA as described [21]. The ELISA was sensitive to TNF-α concentrations in the medium above 40 pg/mL. The ELISA was performed by coating 96-well plates with 6.25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 hr at room temperature. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to the medium, the assay plates were exposed sequentially to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate. Optical density readings were made within 10 min of the addition of the substrate on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter. Appropriate specificity controls were included.

Statistical Analysis

All values are presented as means ± SD. Student's *t*-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of Spirulina on Compound 48/80-induced Systemic Allergic Reaction

To assess the contribution of spirulina in anaphylactic reaction, we first used the *in vivo* model of systemic allergic reaction. We used compound 48/80 as a systemic fatal allergic reaction inducer. After the i.p. injection of compound 48/80 (8 μg/g body weight), the rats were monitored for 1 hr, after which the mortality rate was determined. As shown in Fig. 1, i.p. injection of 200 μL of saline, as a control, induced fatal shock in 100% of each group. When the mice were pretreated with spirulina (0.01 to 1000 μg/g body weight, i.p.) for 1 hr, the mortality with compound 48/80 was reduced dose dependently. Of special note, spirulina inhibited compound 48/80-induced allergic reaction 100% with doses of 100–1000 μg/g body weight (Fig. 1).

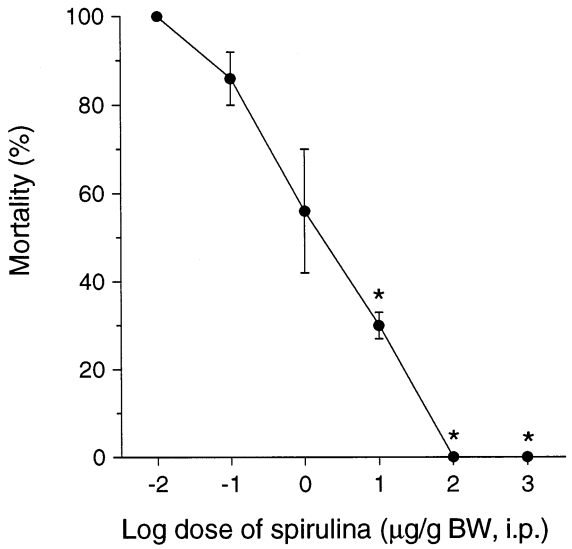

FIG. 1. Effect of spirulina on compound 48/80-induced systemic allergic reaction. Groups of rats were pretreated i.p. with 200 μL saline or spirulina (at various doses) 1 hr before (N = 10/group) the i.p. injection of compound 48/80. Mortality (%) within 1 hr following the injection of compound 48/80 was represented as the number of dead rats × 100/total number of experimental rats. BW = body weight. Each value is the mean ± SD of seven independent experiments. *Significantly different from the saline value, $P < 0.05$.

Effect of Spirulina on PCA

Another way to test allergic reaction is to induce PCA in skin. As described in the experimental procedures, local extravasation was induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Administration of spirulina (100–1000 μg/g body weight, i.p.) produced a marked inhibition of the PCA reaction (Fig. 2).

Effect of Spirulina on Serum Histamine Release

The ability of spirulina to influence compound 48/80-induced serum histamine release was investigated. Spirulina was given in doses of 0.01 to 1000 μg/g body weight 1 hr before the injection of compound 48/80 ($N = 5$/group). While serum levels of histamine were elevated markedly after the injection of compound 48/80 in all groups of rats, the rats injected with spirulina showed a significant reduction in serum histamine levels (Fig. 3).

Effect of Spirulina on Histamine Release from RPMC

The inhibitory effects of spirulina on compound 48/80-induced or IgE-mediated histamine release from RPMC are shown in Fig. 4. Spirulina dose-dependently inhibited compound 48/80-induced or IgE-mediated histamine release at concentrations from 0.001 to 10 μg/mL.

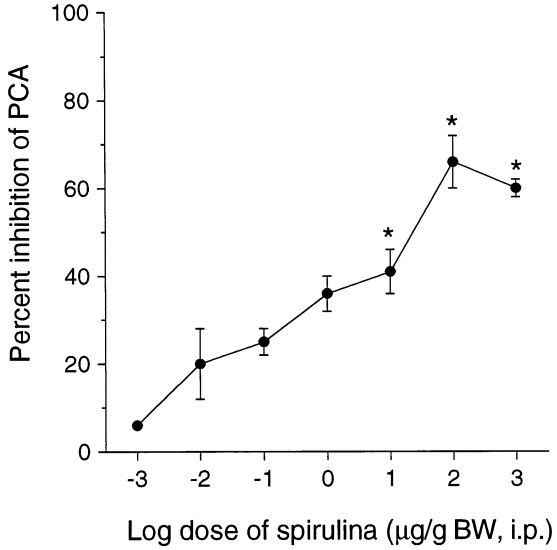

FIG. 2. Effect of spirulina on 48-hr PCA. Spirulina was administered i.p. 1 hr prior to the challenge with antigen (N = 4). All values are expressed as a percentage of the control; 0% = 27.95 μg (amount of dye/site); 100% = 0 μg (amount of dye/site). BW, body weight. Each value is the mean ± SD of four independent experiments. ⋆Significantly different from the saline value, $P < 0.05$.

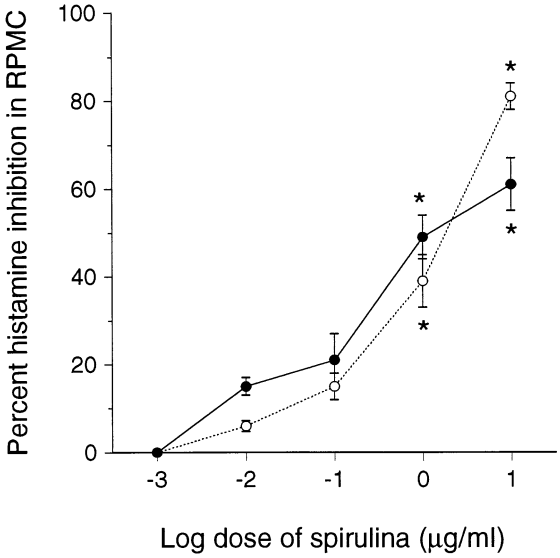

FIG. 4. Effect of spirulina on compound 48/80-induced (●) or IgE-mediated (○) histamine release from RPMC. RPMC (2 × 10^5 cells/mL) were preincubated with spirulina at 37° for 10 min prior to incubation with compound 48/80 or DNP-HSA. All values are expressed as a percentage of the control; 100% = 3.01 ± 0.86 μg/mL. Each value is the mean ± SD of three independent experiments. ⋆Significantly different from the saline value, $P < 0.05$.

Effect of Spirulina on cAMP Level and TNF-α Production

Data in Fig. 5 show the analysis of intracellular cAMP levels. When RPMC were incubated with spirulina at a concentration of 10 μg/mL, the cAMP content increased about 70-fold compared with that of the control cells. It peaked 10 sec after the spirulina was added, and then decreased to basal value about 30 sec later. Spirulina (10 μg/mL) also inhibited IgE-mediated TNF-α production from RPMC (Table 1). No significant cytotoxicity of spirulina on the culture was observed in the concentration used in the experiments, as assessed by trypan blue uptake.

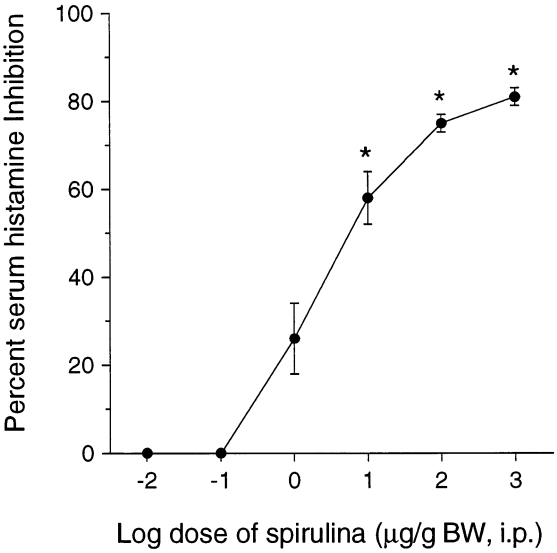

FIG. 3. Effect of spirulina on compound 48/80-induced serum histamine release. Groups of rats were pretreated i.p. with 200 μL saline or spirulina (at various doses) 1 hr before (N = 5/group) the injection of compound 48/80. All values are expressed as a percentage of the control; 100% = 1.97 ± 0.05 ng/mL. BW, body weight. Each value is the mean ± SD of three independent experiments. ⋆Significantly different from the saline value, $P < 0.05$.

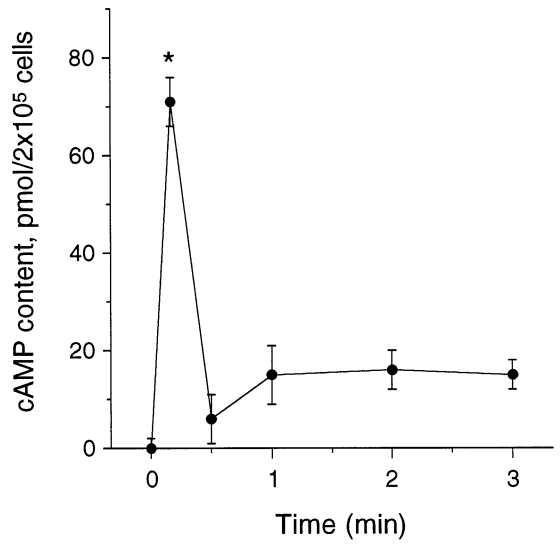

FIG. 5. Time–course of the increase in the cAMP level of RPMC caused by spirulina. RPMC (2 × 10^5 cells/mL) were pretreated with spirulina (10 μg/mL) at 37°. Each value is the mean ± SD of three independent experiments. ⋆Significantly different from the saline value, $P < 0.05$.

TABLE 1. Effect of spirulina on IgE-mediated TNF-α production

Spirulina addition (μg/mL)	Anti-DNP IgE plus DNP-HSA addition	TNF-α production (ng/mL)
None (saline)	−	0.23 ± 0.01
None (saline)	+	0.94 ± 0.04
10	+	0.47 ± 0.06*

RPMC (2×10^5 cells/mL) were sensitized with anti-DNP IgE (1 μg/mL) and incubated for 6 hr in the absence or presence of spirulina before the challenge with DNP-HSA (0.1 μg/mL). TNF-α release into the medium is presented as the mean ± SD of three independent experiments from the RPMC of three rats.

*Significantly different from both saline values, $P < 0.05$.

DISCUSSION

We have demonstrated that spirulina pretreatment profoundly affected compound 48/80-induced systemic allergic reaction and anti-DNP IgE-induced local allergic reaction. Spirulina inhibited the release of histamine induced by specific antigens as well as nonspecific mechanisms from mast cells. We speculate that these results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by spirulina. Qureshi *et al.* [22, 23] have reported that spirulina enhances macrophage phagocytic function and several immunological functions, implying that spirulina supplementation may improve the disease-resistance potential in cats and chickens. These results suggest that spirulina is a source of many biochemicals. There is no doubt that stimulation of mast cells with compound 48/80 or anti-DNP IgE initiates the activation of a signal-transduction pathway that leads to histamine release. Some recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins [24, 25]. The evidence indicates that the protein is G_i-like and that the activation is inhibited by benzalkonium chloride [26]. Tasaka *et al.* [27] reported that compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the membrane permeability increase may be an essential trigger for the release of the mediator from mast cells. Spirulina might act on the lipid bilayer membrane, affecting the prevention of the perturbation being induced by compound 48/80. This is supported by a previous report that benzalkonium chloride and other selective antagonists inhibit the histamine release induced by compound 48/80 [28]. Rats administered spirulina are protected from IgE-mediated cutaneous allergic reaction. The possible mechanism of these effects appears to be related to the activation of adenylate cyclase and a subsequent increase in intracellular cAMP [29]. The intracellular cAMP content of the mast cells, when incubated with spirulina, increased about 70-fold in comparison with that of basal cells (Fig. 5). The mode of action of spirulina is likely related to the prevention of calcium release from the calcium store of mast cells due to elevation of the intracellular cAMP level by inhibition of the cAMP phosphodiesterase. Our data showed that spirulina inhibited anti-DNP IgE-induced TNF-α production. The effect of spirulina on mast cell cytokine production *in vivo* and the relative importance of mast cells as a source of TNF-α during inflammatory and immune responses are important areas for future studies. In conclusion, the results obtained proved that spirulina inhibited the IgE-mediated allergic reaction *in vivo* and *in vitro* in a murine model. Further work should address the possibility that spirulina may also be active in the inhibition of human mast cell degranulation and, therefore, in the treatment of human allergic disorders.

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