

Immunomodulatory Effect of a New Herbal Preparation (HemoHIM) in Cyclophosphamide-treated Mice

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Abstract

The immunomodulatory effect of a new herbal preparation, HemoHIM, on the recovery from leukopenia induced by cyclophosphamide treatment was investigated. The HemoHIM was made up with an addition of the ethanol-insoluble fraction to the total water extract of *Angelica Radix*, *Cnidii Rhizoma* and *Paeonia Radix*. Daily oral administration of 100 mg/kg BW or 500 mg/kg BW HemoHIM accelerated the recovery from cyclophosphamide-induced leukopenia. HemoHIM increased the number of leukocytes and lymphocytes in the peripheral blood when compared with the cyclophosphamide-treated control. Moreover, the suppressed natural killer (NK) cell activity and interferon (IFN)- γ secretion in the cyclophosphamide-treated mice were restored by the administration of HemoHIM. HemoHIM significantly reduced the abnormally heightened ratio of interleukin (IL)-4/IFN- γ and immunoglobulin (Ig)E/IgG2a in the cyclophosphamide-treated mice. These results suggest that HemoHIM accelerates the recovery from leukopenia and alleviates the imbalanced T helper (Th)1/Th2 responses in the cyclophosphamide-treated mice. Additionally, HemoHIM was found to stimulate normal splenocytes to secrete not only Th1 type cytokines such as IFN- γ and IL-2, but also Th2 type cytokine IL-4. In conclusion, our results show that HemoHIM certainly has an influence on the balanced recovery of immune cells and the activation of their activities in the cyclophosphamide-treated mice.

Key words: immune, modulatory, herbal, Th1/Th2 balance, cyclophosphamide

INTRODUCTION

Many herbs or herbal prescriptions consisting of several medicinal plants, in traditional Oriental medicine, have been reputed to promote health, to improve the defense mechanisms of the body and to enhance longevity. These attributes are similar to the modern concept of adaptogenic agents, which are known to afford a protection of the human physiological system against diverse stressors. Based on the related principles, the modulation of the immune responses to alleviate diseases has been of interest for many years. Numerous non-specific immunomodulators have been used, including substances isolated from fungi or microorganisms and from plants. A number of herbs have been said to possess immunomodulatory activity. Some of the herbs and prescriptions have been studied for their immunomodulatory activities in cyclophosphamide-treated animals (1-4).

Cyclophosphamide is a well-known immunosuppressive agent, besides a cytotoxic drug (5). Cyclophosphamide, a multifunctional alkylating agent, is a cytotoxic drug

that interferes with DNA synthesis and has its major pharmacological action on dividing cells. Cyclophosphamide is primarily used to inhibit the growth of some tumors, to treat various autoimmune disorders and immune-mediated inflammatory diseases and to prevent a rejection of organ transplants. Early studies on the immunologic effects of cyclophosphamide demonstrated a decrease in the absolute number of T cells, a reduction in the circulating B cells, a decrease in the synthesis of IgG, and a decrease in the spontaneous proliferation of lymphocytes (6,7). Sasaki et al. (8) demonstrated that a treatment of cyclophosphamide before an epicutaneous immunization with picryl chloride resulted in a marked blood eosinophilia, which was maximal at 13 days. Several researchers have found that cyclophosphamide modulates the differentiation of the Th cell into Th2 cells (9-12). In a recent review, cyclophosphamide was reported to have selective immune effects by suppressing Th1-type responses and enhancing Th2 responses, although a general immunosuppressant that affects both T- and B-cell function (13).

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The differentiation of naive Th cells into Th1 or Th2 cells is known to have important biologic implications for susceptibility or resistance to a particular disease (14, 15). The high response of the Th1 cells is associated with a classical inflammation and autoimmune disease. On the other hand, the high response of the Th2 cells is associated with an allergic inflammation and asthma. A selective differentiation to either subset is established during priming and can be significantly influenced by a variety of factors.

A new herbal preparation, HemoHIM, is designed to protect the self-renewal tissues and promote the recovery of the immune system against oxidative stress such as irradiation. HemoHIM was prepared by adding its polysaccharide fraction to a hot water extract of herb mixture consisting of *Angelica Radix*, *Cnidii Rhizoma* and *Paeonia Radix*. In previous studies, HemoHIM was tested for efficacy as a radioprotective and immunomodulatory agent (16,17).

In this study, we investigated the effect of HemoHIM on an accelerated and balanced recovery of immune cells in cyclophosphamide-treated immunosuppressed mice.

MATERIALS AND METHODS

Mice

Specific pathogen-free ICR and C57BL/6 (H-2^b) mice, 8 to 9 weeks old, were purchased from the Orient Inc. (Charles River Technology; Seoul, Korea). The mice were housed in polycarbonate cages, and were fed a standard animal diet and water *ad libitum*. Research was conducted according to principles enunciated in the 'Animal Care Act', prepared by the Ministry of Agriculture and Forestry, Republic of Korea.

Preparation of HemoHIM

A mixture of 3 edible medicinal herbs, *Angelica Radix* (root of *Angelica gigas* Nakai), *Cnidii Rhizoma* (rhizome of *Cnidium officinale* Makino), and *Paeonia Radix* (root of *Paeonia japonica* Miyabe), was decocted for 4 hours in boiling water to obtain a total extract (HIM-I). A part of HIM-I was fractionated into an ethanol-soluble fraction and an ethanol-insoluble polysaccharide fraction by a precipitation in 80% ethanol. HemoHIM was prepared by adding the polysaccharide fraction to the other part of HIM-I.

Antibodies and standards for the enzyme-linked immunosorbent assay (ELISA)

For the IFN- γ measurements, clone R4-6A2 was used as the capture Ab, and biotin-labeled XMG1.2 as the detecting Ab. For IL-4, clone BCD4-1D11 was the cap-

ture Ab and biotin-labeled BVD6-24G2 was the detecting Ab. Biotin-conjugated anti-IgG2a antibody and biotin-conjugated anti-IgE antibody were used as the detecting Ab for the measurement of the immunoglobulin. These Abs as well as recombinant IFN- γ and IL-4 were purchased from PharMingen (San Diego, CA, USA).

Cyclophosphamide treatment and HemoHIM administration

The animals were intraperitoneally (i.p.) injected with cyclophosphamide (Sigma-Aldrich Co., St. Louis, MO, USA) at a dose of 200 mg/kg body weight (BW) on day 0 before oral administration of HemoHIM. HemoHIM was orally administered at a dose of 100 mg/kg BW or 500 mg/kg BW per day from day 0 just after a cyclophosphamide treatment for a period according to each experimental procedure.

Counting of the peripheral blood cells

HemoHIM was orally administered daily just after cyclophosphamide treatment for 43 days. Peripheral whole blood was collected from the retro-orbital veins of mice at the time points indicated in Fig. 1 after the cyclophosphamide treatment. Leukocytes and lymphocytes in the peripheral blood were automatically counted by a hematology analyzer (HEMAVET 850⁺, CDC Technologies, Inc., USA). The average value for each group was obtained from twenty mice.

Preparation of the splenic lymphocytes

Spleens were removed aseptically from the mice, and single cell suspensions were prepared by mincing the spleens. The splenic lymphocytes were prepared by a density gradient centrifugation on a Ficoll-Hypaque solution (Sigma-Aldrich Co., St. Louis, MO, USA). All the cell suspensions were maintained in RPMI (Rosewell Park Memorial Institute) 1640 medium (GIBCO BRL, Paisley, UK) supplemented with 20 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 100 U/mL penicillin, 50 μ g/mL streptomycin, 0.05 mM 2-mercaptoethanol, 1% non-essential amino acid and 10% fetal bovine serum (FBS). All the reagents were purchased from GIBCO BRL (Paisley, UK).

The measurement of the NK cell activity by the ⁵¹Cr-release assay

HemoHIM was orally administered for 3 weeks after cyclophosphamide treatment. 4 weeks after the cyclophosphamide treatment, spleen lymphocytes were prepared and the NK cell activity was determined by the ⁵¹Cr-release assay. In brief, YAC-1, a Moloney virus-induced mouse T-cell lymphoma of A/Sn origin, was used as target cells. 2×10^6 YAC-1 cells were labeled with 40

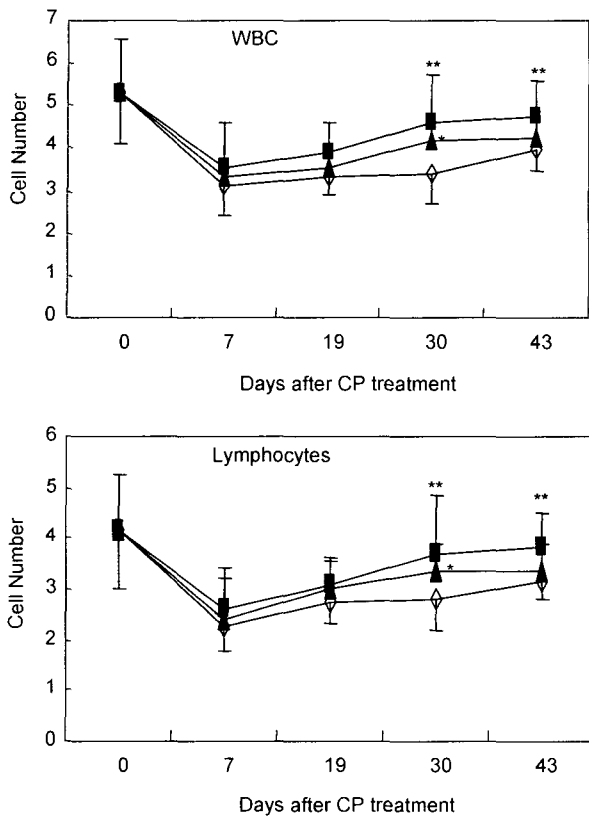


Fig. 1. Acceleration of the recovery from cyclophosphamide (CP)-induced leukopenia in peripheral blood by HemoHIM. ICR mice were injected with CP (200 mg/kg BW) on day 0. HemoHIM was orally administered daily just after the CP treatment until the blood sampling. \diamond , CP-treated mice; \blacksquare and \blacktriangle , 100 mg/kg and 500 mg/kg BW HemoHIM administered mice after CP treatment, respectively. The results are expressed as the mean \pm SD of 20 mice/group. * $p < 0.1$, ** $p < 0.05$.

μ Ci of sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) for 60 min at 37 $^\circ\text{C}$ in a water bath. After a labeling, the cells were washed three times with HBSS and resuspended at a concentration of $2 \times 10^5/\text{mL}$ in the complete medium. Spleen lymphocytes prepared by the above method were used as effector cells. Effector and ^{51}Cr -labelled target cells were dispensed into round-bottom microtiter plate wells (Corning, New York, USA) in triplicates, at effector to target ratios of 100:1 and 50:1. Plates were centrifuged at 500 rpm for 3 min and incubated for 4 hours at 37 $^\circ\text{C}$ in a humidified CO_2 incubator. After 4 hours, the plates were centrifuged at 1500 rpm for 10 min and 100 μL of the supernatants were collected for radioactivity counts in a γ -counting system (Wallac, Minnesota, USA). Spontaneous or maximal release was determined by the target cells with the added medium alone or 1% Triton X-100, respectively. Percentage of cytotoxicity, as measured by a specific ^{51}Cr -release, was calculated by using the formula: $(\text{cpm experimental} - \text{cpm spontaneous}) / (\text{cpm maximal} - \text{cpm spontaneous}) \times 100$.

In vitro stimulation of spleen lymphocytes for cytokine productions and cytokine measurements by ELISA

HemoHIM was orally administered to the mice for 3 weeks after cyclophosphamide treatment. 4 weeks after the cyclophosphamide treatment, the spleen lymphocytes were prepared from the mice and were cultured at 2×10^6 cells/mL/well in 24-well plates (Corning, New York, USA) pre-coated with 1 μg /well of anti-CD3 mAb and 1 μg /well of anti-CD28 mAb. After 2 days, the culture supernatants were harvested, and the amounts of IFN- γ and IL-4 were determined using a sandwich ELISA. In brief, microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with a capture antibody at a concentration of 0.2 μg /well in a 0.1 M sodium carbonate buffer (pH 9.5) overnight at 4 $^\circ\text{C}$. The unbound antibodies were removed by washing with PBS containing Tween 20 (washing buffer) three times. The plates were blocked with 10% FBS in HBSS (Hank's balance salt solution; GIBCO BRL, Paisley, UK). After washing, diluted samples were added to the first antibody-coated well and then incubated for 2 hr at room temperature. After another washing, the biotin-labeled detecting antibody (0.2 μg /well) was added to the well, and incubated for 1 hr at room temperature. The plate was washed five times with a washing buffer. Diluted streptavidin-HRP (horse radish peroxidase) was added to each well and incubated at room temperature for 1 hr. After washing each well at least eight times, each well was incubated with 200 μL of a TMB substrate solution (BD PharMingen, San Diego, CA, USA) for 5 ~ 20 min and 50 μL of the stop solution (1 M phosphoric acid; Sigma-Aldrich Co., St. Louis, MO, USA) was added to each well. The absorbance was measured by a microplate reader at a wavelength of 450 nm with reference at 570 nm.

Immunization and serum preparation and the measurement of antigen-specific immunoglobulin

HemoHIM was orally administered to the mice for 2 weeks after a cyclophosphamide treatment. 2 weeks after the cyclophosphamide treatment, the mice were immunized with DNP-KLH (Calbiochem, San Diego, California, USA) by an *i.p.* injection (0.2 mL) of an emulsion of equal volumes of DNP-KLH in a phosphate buffered saline (PBS) and complete Freund's adjuvant (CFA; Calbiochem, San Diego, California, USA). Finally each animal received 100 μg of protein, 1 week after immunization, whole blood was collected from the retro-orbital veins of the mice and allowed to clot at room temperature. Serum was separated by a centrifugation and stored at -70 $^\circ\text{C}$ for further tests. The antigen-specific IgG2a and IgE in the serum were measured by ELISA,

as described above.

Analysis of the cytokine mRNA expression in the spleen cells

Total RNA was purified from the spleen cells using RNazol B (Tel-Test, Inc., Friendswood, TX, USA). mRNA analysis was performed by a RT-PCR. cDNA synthesis from 2 µg total RNA was performed in a 20 µL reaction using a M-MLV (Moloney-murine leukemia virus) reverse transcriptase (Promega, Madison, WI, USA). cDNAs of interest were amplified from 1 µL RT product per PCR using Taq DNA polymerase. The primers were as follows: β -actin, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3', 500bp; IL-2, 5'-AACAGCGCACCCACTTCAA-3' and 5'-TTGAG-ATGATGCTTTGACA-3', 442bp; IFN- γ , 5'-AACGCT-ACACACTGCATCT-3' and 5'-TGCTCATTGTAATG-CCTTG-3', 342bp; IL-4, 5'-TAGTTGTCATCCTGCTC-TT-3' and 5'-CTACGAGTAATCCATTTGC-3', 404bp. PCRs were optimized such that the number of cycles that were used allowed for an analysis of the products within the linear part of the amplification reaction. Amplification was performed for 20s at 95°C, 30s at 60°C, and 40s at 72°C in a thermal cycler (UNO II; Biometra, Goettingen, Germany). PCR products were resolved on 1% agarose gel.

Statistical analysis

Statistical significance of the data was determined by Student's *t*-test. Differences with a *p* value of less than 0.05 were taken as significant, and considerable with a *p* value of less than 0.1.

RESULTS AND DISCUSSION

Recovery from cyclophosphamide-induced leukopenia in mice by HemoHIM administration

When ICR mice were orally administered 100 or 500 mg/kg BW of HemoHIM once a day, starting just after a cyclophosphamide treatment, recovery of the leukocyte and lymphocyte numbers in the peripheral blood was accelerated (Fig. 1). In the cyclophosphamide-treated mice, the populations of both cells in the peripheral blood were significantly decreased and did not recover until 30 days. However, in the HemoHIM administered mice after the cyclophosphamide treatment, the number of leukocytes and lymphocytes were increased significantly ($p < 0.05$) at 30 days and 43 days after the cyclophosphamide treatment, compared with the mice treated with cyclophosphamide alone.

The effect of HemoHIM on NK cell activity in the cyclophosphamide-treated mice

It is reported that the number and activity of

radioresistant NK cells are higher within 10 days after irradiation when compared with normal mice, but the activity of the NK cells goes down from 2 weeks after an irradiation (18). We presumed that the cyclophosphamide treatment and whole body irradiation exert similar effects on NK cell activity. Therefore, the unfractionated spleen lymphocytes from mice 4 weeks after cyclophosphamide treatment were used as responder cells. NK cell activity was extremely reduced in the cyclophosphamide-treated mice (Fig. 2). However, the reduced NK cell activity was restored by the HemoHIM administration in the cyclophosphamide-treated mice. Because the NK cells are activated by cytokines such as IFN- γ , this should be evaluated in the context of cytokine secretion in the spleen lymphocytes of the cyclophosphamide-treated mice.

The effect of HemoHIM on the production of IgG2a and IgE in the cyclophosphamide-treated mice

To investigate the differentiation of helper T cells in the cyclophosphamide-treated mice, we examined the IgG2a and IgE production against a specific antigen, DNP-KLH, through the assistance of the Th1 and Th2 cells, respectively. While the IgG2a level was lower, the IgE level was much higher in the cyclophosphamide-treated mice when compared with the normal mice (Fig. 3). From this data, we assumed that the helper T cells preferentially differentiate into Th2 following a cyclophosphamide treatment. On the other hand, in the HemoHIM administered cyclophosphamide-treated mice, the IgG2a secretion was enhanced and especially, the IgE secretion was reduced to a normal level (Fig. 3). This result suggests that the HemoHIM administration modulates the

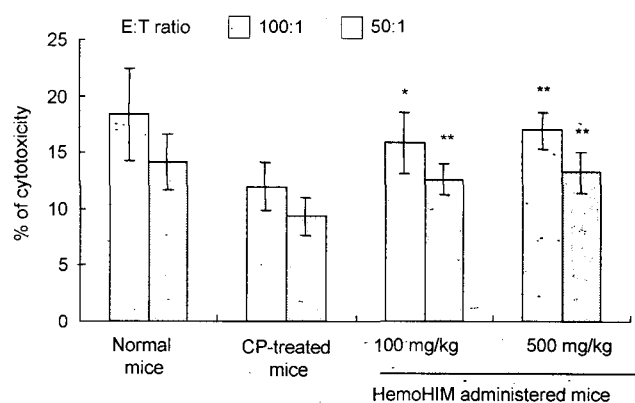


Fig. 2. Effect of HemoHIM on the restoration of NK cell activity in cyclophosphamide (CP)-treated mice. C57BL/6 mice were injected with CP (200 mg/kg BW). HemoHIM was orally administered daily just after the CP treatment for 3 weeks. 4 weeks after the CP treatment, the NK cell activity of the mice of each group was measured by the ^{51}Cr -release assay. The data are expressed as the mean \pm SD of 6 mice/group. * $p < 0.05$, ** $p < 0.01$.

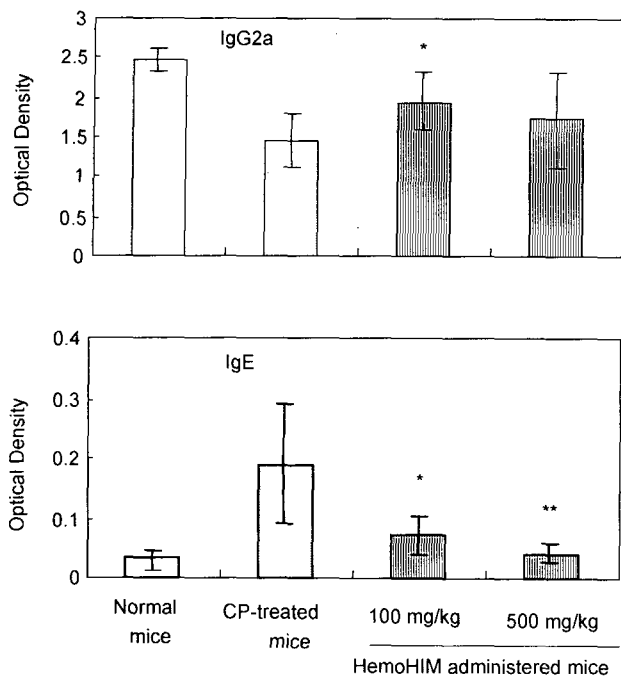


Fig. 3. Effect of HemoHIM on the production of an antigen specific IgG2a and IgE in cyclophosphamide (CP)-treated mice. C57BL/6 mice were injected with CP (200 mg/kg BW). HemoHIM was orally administered daily just after CP treatment for 2 weeks. The mice were immunized with DNP-KLH (100 μ g) i.p. at 2 weeks after the CP treatment. The mice were bled from retro-orbital veins 1 week after the immunization. The serum anti-DNP IgG2a and IgE concentrations were measured by an indirect ELISA. Data are expressed as the mean \pm SD of 6 mice/group. * p <0.05, ** p <0.01.

shift differentiation of naive helper T cells in the cyclophosphamide-treated mice.

The effect of HemoHIM on the secretion of cytokines in the cyclophosphamide-treated mice

Cyclophosphamide treatment decreased the IgG2a secretion, but it increased the IgE secretion, and this situation was modulated by the HemoHIM administration (Fig. 3). Therefore, the effects of HemoHIM on the Th1 or Th2 type cytokine secretion were investigated in the cyclophosphamide-treated mice. The spleen lymphocytes were prepared from mice 4 weeks after cyclophosphamide treatment, and were stimulated by anti-CD3/CD28 antibody *in vitro*, and then IFN- γ and IL-4 in the culture supernatant were measured by ELISA (Fig. 4). In the cyclophosphamide-treated mice, the secretion of IFN- γ was markedly decreased when compared with the normal mice (Fig. 4A). The oral administration of 100 mg/kg BW and 500 mg/kg BW HemoHIM restored the reduced secretion of IFN- γ in the cyclophosphamide-treated mice (p <0.1). The recovery of the IFN- γ secretion may be related to the enhancement of the NK cell activity (Fig. 2). IgE secretion is induced by

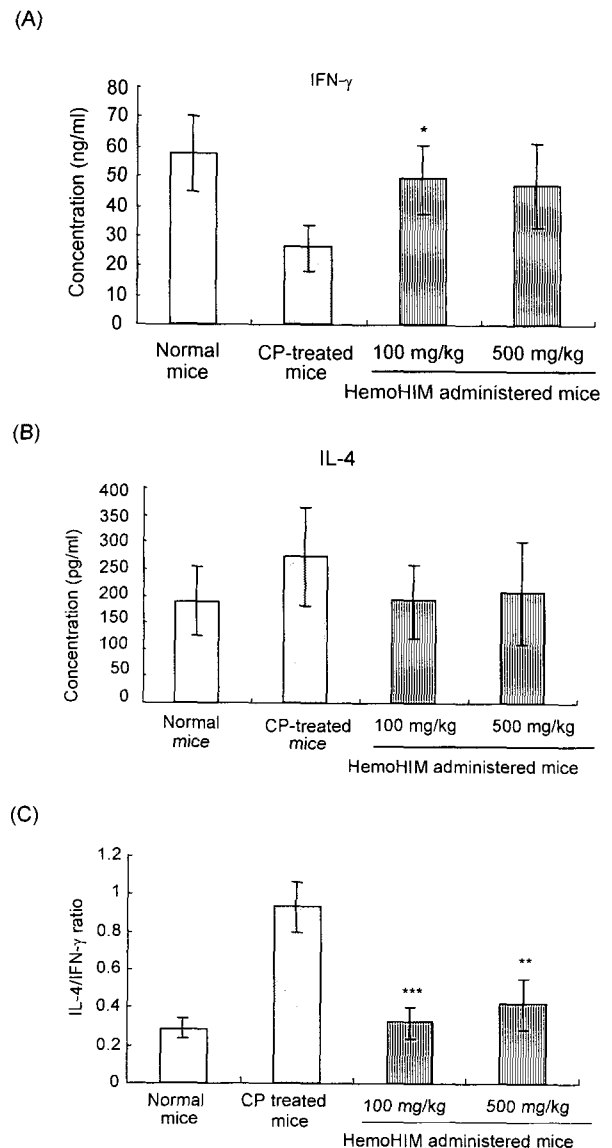


Fig. 4. Effect of HemoHIM on the secretion of IFN- γ and IL-4 and the ratio of IL-4/IFN- γ in the splenic lymphocytes of the cyclophosphamide (CP)-treated mice. The C57BL/6 mice were injected with CP (200 mg/kg BW). HemoHIM was orally administered daily just after the CP treatment for 3 weeks. 4 weeks after the CP treatment, spleen lymphocytes were prepared and stimulated *in vitro* by the pre-coated anti-CD3/CD28 antibody for 18 hours and then cytokines in their culture supernatant were measured by ELISA. (A) IFN- γ secretion, (B) IL-4 secretion, (C) IL-4/IFN- γ ratio. The results are expressed as the mean \pm SE of 6 mice/group. * p <0.1, ** p <0.01, *** p <0.003.

an over production of IL-4 from Th2 cells (15,19-22). As expected, the secretion of IL-4 in the cyclophosphamide-treated mice was increased when compared with the normal mice. The oral administration of HemoHIM reduced the heightened IL-4 secretion in the cyclophosphamide-treated mice (Fig. 4B). Also, IL-4 is known as a cytokine which antagonizes several biolog-

ical actions of IFN- γ secreted by the Th1 cells (23,24). Specifically, the biological action of IL-4 depends on the ratio to IFN- γ . So, we calculated the ratio of IL-4 to IFN- γ as shown in Fig. 4C. In the cyclophosphamide-treated mice, the ratio of IL-4/IFN- γ was much higher when compared with the normal mice. The oral administration of HemoHIM reduced the abnormally increased ratio of IL-4/IFN- γ to the level of the normal mice.

The effect of HemoHIM on the expression of cytokine genes in the splenocytes

The effects of HemoHIM on the Th1 or Th2 type cytokine secretion were investigated in normal splenocytes. Naive T cells differentiate into various effector lineages to orchestrate effective immune responses. The best characterized lineages are the Th1 cells, producing IFN- γ and IL-2, and the Th2 cells, producing IL-4, IL-5 and IL-13 (25). The ratio of Th1 to Th2 cells exerts important effects on the balance in the cellular and humoral immunities. If such a balance were broken, the body would acquire a disease such as an autoimmune disease or an allergic disease. HemoHIM treatment was found to increase the secretion of not only IFN- γ and IL-2, but also IL-4 in the splenocytes (Fig. 5). Therefore, we concluded that the equilibrium stimulation of Th1 cells and Th2 cells by HemoHIM rather than the shifted stimulation is very important for effective immune responses.

The role of HemoHIM in a balanced recovery of the immune responses after a cyclophosphamide treatment

We took an interest in the balanced responses of the

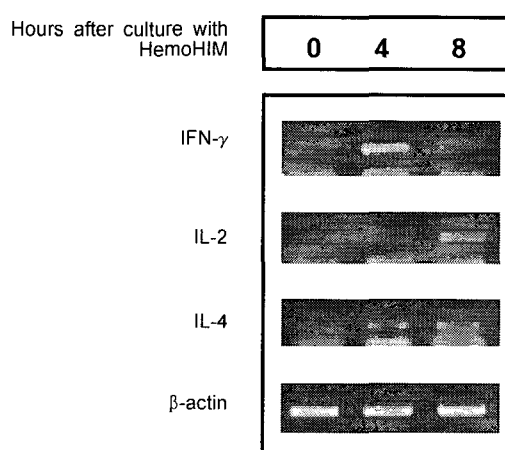


Fig. 5. Cytokine expression in the splenocytes cultured with HemoHIM. Total spleen cells of the normal mice were cultured with HemoHIM at a final concentration of 300 μ g/mL, and then the total RNA was isolated from the cells after 4 and 8 hour incubations. Cytokine mRNAs induced by HemoHIM were measured by RT-PCR. 0, untreated; 4, treated with HemoHIM for 4 hours; 8, treated with HemoHIM for 8 hours.

immune cells after recovery from cyclophosphamide treatment. Th cells are known to be divided into two types of effector cells based on their functional capabilities and the profile of the cytokines they produce (14,15,24,25). Th1 cells synthesize IL-2, IFN- γ and lymphotoxin, and mediate delayed-type hypersensitivity (DTH) reactions as well as suppressing an IgE response, due to the secretion of IFN- γ (19,23). Conversely, Th2 cells secrete IL-4 and IL-5 and then augment the IgE production, which is mediated by IL-4 (20), but they do not secrete IFN- γ and IL-2. Although cyclophosphamide is considered to be a general immunosuppressant, it has a differential immunomodulatory effect on the immune system that favors Th2 type responses (11,12). In the study of Karni et al. (12), cyclophosphamide was associated with an increased percentage of the CCR4⁺CD4⁺T cells and with increased percentages of the IL-4-producing CD4⁺T cells and the IL-4-producing CCR4⁺CD4⁺T cells. An increased IL-4 production in cyclophosphamide-treated patients has been described previously by several researchers (9,11). In general, Th2 cells can down-regulate the inflammation associated with a Th1-mediated immune response (14). However Th2 cells are the principal effectors of the IgE-mediated immune response. So, an excessive response of Th2 cells can induce an allergic inflammation. Our present study showed that in the cyclophosphamide-treated mice, the NK cell activity and the IFN- γ and IgG2a secretions are reduced and the IL-4 and IgE secretions are increased. These data suggest that cyclophosphamide induces a preferential differentiation of Th cells into Th2 cells. However HemoHIM alleviated the imbalanced Th1/Th2 responses. In conclusion, our results show that HemoHIM certainly has an influence on the balanced recovery of the immune cells and the restoration of their activities in cyclophosphamide-treated mice.

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