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Restoration of the Immune Functions in Aged Mice by Supplementation with a New Herbal Composition, HemoHIM

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The effect of a new herbal composition, HemoHIM, on immune functions was examined in aged mice, in which various immune responses had been impaired. The composition HemoHIM was prepared by adding the ethanol-insoluble fraction to the total water extract of a mixture of three edible herbs, Angelica Radix, Cnidium Rhizoma and Paeonia Radix. Supplementation to the aged mice with HemoHIM restored the proliferative response and cytokine production of splenocytes with a response to ConA. Also, HemoHIM recovered the NK cell activity which had been impaired in the aged mice. Meanwhile aging is known to reduce the Th1-like function, but not the Th2-like function, resulting in a Th1/Th2 imbalance. HemoHIM restored the Th1/Th2 balance in the aged mice through enhanced IFN-γ and IgG2a production, and conversely a reduced IL-4 and IgG1 production. It was found that one factor for the Th1/Th2 imbalance in the aged mice was a lower production of IL-12p70. However, HemoHIM restored the IL-12p70 production in the aged mice. These results suggested that HemoHIM was effective for the restoration of impaired immune functions of the aged mice and therefore could be a good recommendation for immune restoration in elderly humans. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: aging; immunomodulatory; Th1/Th2 balance; herbal medicine; immune function.

INTRODUCTION

It is well known that many biological functions change with age. The majority of the changes are undesirable and often subject the aged to a higher risk of developing diseases. The immune system, in particular, is sensitive to age-related changes (Solana *et al.*, 1991; Miller, 1996).

Almost all of the various cell types of the immune system experience a change with aging; however, the most prominent change occurs in the T cells. Declined T cell function is believed to be the central defect in immune senescence (Globerson, 1995; Pawelec, 1995). Impaired T cell function has been well documented from numerous animal and human studies (Miller, 2000; Linton and Dorshkind, 2004). It is well recognized that T cell proliferation declines with aging, whether measured *in vitro*, in cultures stimulated with mitogens, antigens, or anti-CD3 antibody, or *in vivo* as delayed-type hypersensitivity (DTH) responses, antibody production to T cell-dependent antigens, or as manifested in the increased incidence of infectious disease. In both mice and humans, IL-2 production, as well as its receptor expression, declines with age, resulting in a diminished proliferation of the T cells. Like the data in the present study, most data have shown a senescent decrease in the NK function, mainly in murine cells (Bocchieri *et al.*, 1988; Hirokawa *et al.*, 1994) and in the antibody-dependent cellular cytotoxicity (ADCC) (Mysliwska *et al.*, 1985). There are contradictory reports on age-related changes in cytotoxic activity, especially as regards NK function which has been reported to increase (Krishnaraj and Blandorf, 1988) or to remain unchanged (Ligtharti *et al.*, 1989) with age.

Another serious problem is that aging involves a shift towards a dominance of a Th2 related response (Shearer, 1997; Rink *et al.*, 1998; Albers *et al.*, 2005; Alberti *et al.*, 2006). The impact of age on cytokine production was examined in both animal and human models in an effort to elucidate the possible mechanisms of age-associated changes in immunity. Murine models of aging demonstrated that there was an age-associated dysregulation in the cytokine production, as evidenced by the consistently decreased production of IL-2 (Shearer, 1997) and a generally increased production of IL-4 (Hobbs *et al.*, 1993; Albright *et al.*, 1995). Also, up-regulated IL-4, IL-5 and IL-10 production due to aging have been reported (Rink *et al.*, 1998). These results suggest that aging is associated with a shift that can be at least partially attributed to a change in the phenotypic profile of T cells.

Since Harman proposed in 1956 that oxygen free radicals were tightly related to the age-dependent impairment in cell function (Harman, 1956), growing evidence from scientific studies supports the fact that aging is associated with progressive dysregulation of the immune responses as a result of increased oxidative stress (Miquel, 1998; Barja, 2004).

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A new herbal composition, HemoHIM, was designed by adding its polysaccharide fraction to a hot water extract of a herb mixture consisting of Angelica Radix, Cnidium Rhizoma and Paeonia Radix to protect the self-renewal tissues and to promote recovery of the immune system against oxidative stress such as irradiation (Park *et al.*, 2005; Jo *et al.*, 2005). In traditional Oriental medicine, many herbs or herbal prescriptions consisting of several medicinal plants have been reputed to promote health, to improve the defense mechanisms of the body and to enhance longevity. Therefore, it was assumed that HemoHIM would improve the health of an individual against the progression of aging as a result of oxidative stress. These three herbs are listed as raw materials in the Korea Food Code. Also, HemoHIM has been proven to be safe during a long-term administration test (data not shown).

The present study investigated the effect of HemoHIM supplementation on immune restoration in aged mice.

MATERIALS AND METHODS

Animals. In this study, 2–3-month-old and 12-month-old female C57BL/6 (H-2^b) mice were used as young and aged mice (The Orient Inc; Charles River Technology, Seoul, Korea), respectively. The mice were housed in polycarbonate cages and fed with a standard animal diet and water *ad libitum*. Research was conducted according to the principles enunciated in the 'Animal Care Act', prepared by the Ministry of Agriculture and Forestry, Republic of Korea.

The experimental group was intubated with Hemo-HIM at a final concentration of 100 mg/kg body weight for 3 weeks, while the control group received only water.

Preparation of HemoHIM. A mixture of three 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 h 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 precipitation in 80% ethanol. HemoHIM was prepared by adding the polysaccharide fraction to the other part of HIM-I.

Preparation of lymphocytes in the spleen. Spleens were removed aseptically from the mice and a single cell suspension was prepared by mincing the spleen. The spleen 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 the RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2×10^{-2} M HEPES buffer, 2×10^{-3} M L-glutamine, 1×10^{-3} M pyruvate, 100 U/mL penicillin, 50 µg/mL streptomycin, 5×10^{-5} M of 2-mercaptoethanol and 1% nonessential amino acid. All the supplements as well as RPMI 1640 were purchased from Gibco BRL (Grand Island, USA).

Lymphocyte proliferation assay. To measure cell proliferation, the ^{3}H-thymidine incorporation assay was

performed. The lymphocytes were adjusted to 2×10^5 cells/well. Aliquots of 200 µL were dispensed in plates of 96 wells and 20 µL of ConA (1 µg/mL) or PBS (negative control) was added. After 48 h of incubation, 1.5 µCi of ^{3}H-thymidine (Amersham Pharmacia Biotech, Kangnam-ku, Seoul, Korea) was added to each well, followed by 4 h incubation. The cells were then harvested onto glass-fiber filters by using a cell harvester (Inotec Biosystems Int, Rockville, MD, USA). Radiolabeled thymidine uptake was measured in a β-liquid scintillation counter (Beckman Canada, Mississauga, ON, USA). The results were calculated as arithmetic means of the cpm in three individual wells. The stimulation index (SI) was calculated as follows:

SI = cpm of stimulated cells/cpm of negative control cells

Assay for NK cell activity. The Molony virus induced leukemia cell line, YAC-1 (TIB-160), was obtained from ATCC (Rockville, MD, USA). YAC-1 tumor targets were labeled with ^{51}Cr-sodium chromate (Amersham Pharmacia Biotech, Kangnam-ku, Seoul, Korea) at a dose of 40 µCi/10^6 cells for 60 min. The cells were washed three times in HBSS (Hank's Balanced Salt Solution, Sigma-Aldrich Co.) and resuspended to a final concentration of 2×10^5 cells/mL. Two thousand target cells and 10^6 spleen effector cells were plated into the wells of a 96-well U bottom plate. The plates were then incubated at 37 °C for 4 h in humidified air containing 5% CO_2. Following centrifugation at $350 \times$ **g** for 10 min, 100 µL of the supernatant was harvested from each well and counted for 1 min in a gamma counter (Wallac, Wellesley, MA, USA). The percent lysis was calculated as follows:

% lysis = {[cpm (experimental) − cpm (spontaneous)]/ [cpm (maximum) − cpm (sponstaneous)]} × 100.

Condition for a cytokine production *in vitro*. The unfractioned spleen lymphocytes (5×10^6/well) obtained from the mice were stimulated with concanavalin A (ConA) at 1 µg/mL or ovalbumin (OVA) at 500 µg/ well for 2 or 3 days, respectively.

Immunization and serum preparation. Mice were immunized with DNP-KLH (Calbiochem, San Diego, California, USA) by an i.p. injection of an emulsion of equal volumes of DNP-KLH in a phosphate buffered saline (PBS) and a complete Freund's adjuvant (CFA; Calbiochem, San Diego, California, USA). Finally each animal received 100 µg of protein. To detect an immunoglobulin against an antigen, whole blood was collected from the retro-orbital veins of the mice at 1 week after immunization and then the blood was allowed to clot at room temperature. Serum was separated by a centrifugation and stored at −70 °C for further testing.

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-labelled XMG1.2 was the detecting Ab. For the IL-4, clone BCD4-1D11 was the capture Ab, and biotin-labelled BVD6-24G2 was the detecting Ab. For the IL-2, clone JES6-1A12 was used as the capture Ab, and biotin-labelled JES6-5H4 was the detecting Ab. For the heterodimer IL-12 (IL-12p70), clone 9A5 was the

capture Ab, and biotin-labeled C17.8 was the detecting Ab. Biotin-conjugated anti-IgG2a, anti-IgG1 and anti-IgE antibody were used to measure the immunoglobulin level. All the antibodies as well as the recombinant IFN-γ, IL-2, IL-4 and IL-12p70 were purchased from BD PharMingen (San Diego, CA, USA).

Antigen specific immunoglobulin and cytokine determination from ELISA. Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with a capture antibody (purified antibody) at a concentration of 2 µg/well in a 0.1 M bicarbonate buffer (pH 8.6) overnight at 4 °C. The unbound antibody was removed by washing with PBS containing Tween 20 (washing buffer) three times. The plates were blocked with 1% BSA in PBS. After washing, the diluted samples were added to the first antibody-coated well and incubated for 2 h at room temperature. After washing, the biotin-labeled detecting antibody (2 µg/well) was added to the well, and incubated for 1 h at room temperature. The plate was washed five times with washing buffer. Diluted streptavidin-HRP (horseradish peroxidase) was added to each well and incubated at room temperature for 30 min. After washing each well at least eight times, each well was incubated with 200 µL of a TMB substrate solution for 10–30 min and 50 µL of the stopping solution was added to each well. The fluorescence intensity was measured by a microplate reader at a wavelength of 450 nm with a reference at 570 nm.

Statistical analysis. Data were expressed as mean ± SD and statistical significance was analysed by using Student's *t*-test. Differences with a value of $p < 0.05$ were taken as significant, and considerable with a p value of less than 0.1.

RESULTS

Restorative effect of HemoHIM on the responses of lymphocytes to mitogen in aged mice

The important and often investigated property of lymphocytes is their lymphoproliferative response to mitogens. This function, which has been widely studied in aged animals, is known to decrease with age (De la Fuente *et al.*, 1992). As shown in Fig. 1A, the proliferation of spleen lymphocytes decreased in the aged mice, showing values significantly lower ($p < 0.001$) than those of the young adult mice. On the other hand, aged mice fed with HemoHIM for 3 weeks showed higher stimulation indexes of the spleen lymphocytes than those found in the aged control mice ($p < 0.1$). However, the values from the aged mice fed with HemoHIM were lower than those of the young adult mice.

The cytokines produced when the lymphocytes were stimulated by ConA were measured. Previous studies have shown that lymphocytes from the elderly produced significantly less IL-2 and IFN-γ, known as the most important T cell growth factors, than the lymphocytes from young adults (Gillis *et al.*, 1981; Born *et al.*, 1995; Huang *et al.*, 1992; Sindermann *et al.*, 1993; Kita *et al.*, 1991; Paganelli *et al.*, 1996). As shown in Fig. 1(B), the IFN-γ and IL-2 secretion levels decreased in the aged mice, showing values significantly lower than those of
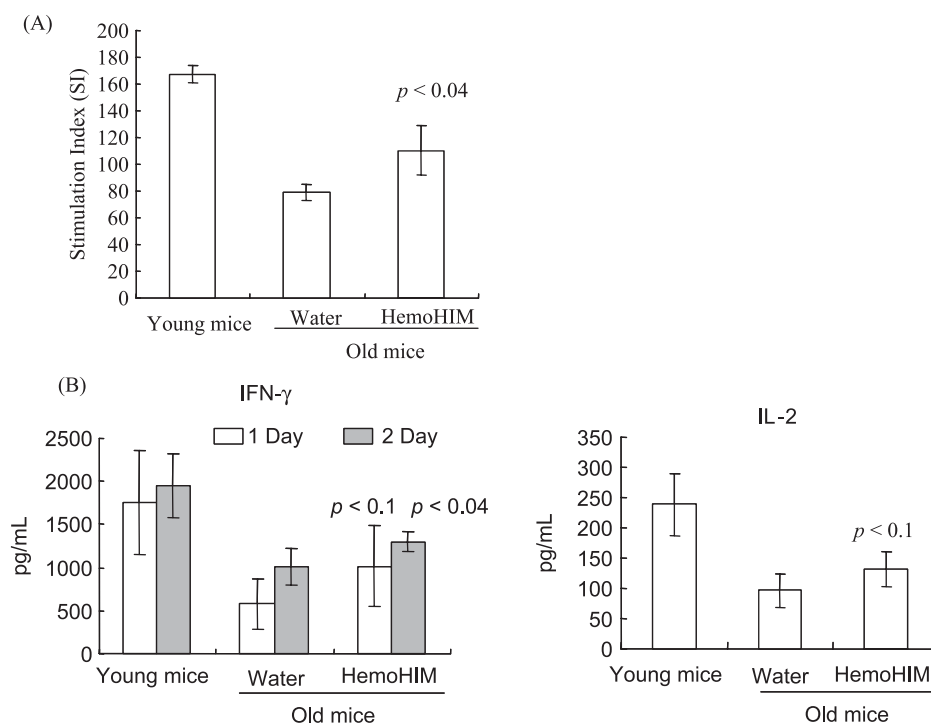

Figure 1. Restoration of the lymphocyte responses to mitogen by HemoHIM in the aged mice. (A) Spleen cells (2 × 10⁵ cells/well) obtained from young adult or aged mice were cultured with 1 µg/mL ConA. On day 2 after incubation, the proliferation of the lymphocytes was determined by the ³H-thymidine incorporation assay. There were five mice in each group. (B) Spleen cells (2 × 10⁶ cells/well) obtained from the young adult or aged mice were stimulated with 1 µg/mL ConA *in vitro* for 24 or 48 h cultures. The levels of IFN-γ and IL-2 in the culture supernatants were measured by an indirect ELISA. There were eight mice in each group. Data show the mean ± SD. * p values mean a significant difference compared with the old mice control.
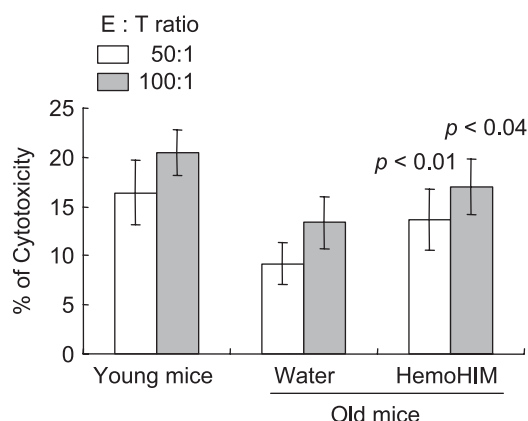
Figure 2. Recovery of the NK cell activity by HemoHIM in the aged mice. Spleen lymphocytes obtained from the young adult or aged mice were co-incubated with ^{51}Cr-labeled YAC-1 (H-2^a) cells. After 4 h of incubation, the cytotoxicity of NK cells was determined by measuring the radioactivity of ^{51}Cr released in the culture supernatant. There were eight mice in each group. Data show the mean ± SD. * p values mean a significant difference compared with the old mice control.

the young adult mice. On the other hand, aged mice fed with HemoHIM for 3 weeks showed restoration of the IFN-γ and IL-2 secretion levels.

HemoHIM recovers the declined NK cell activity in aged mice

Age-associated alterations in the number and function of NK cells have been reported (Mocchegiani and Malavolta, 2004; Plackett *et al.*, 2004). As expected and reported, the NK cell activity of the lymphocytes from the aged mice was significantly decreased when compared with that of the young adult mice (Fig. 2). However, mice fed with HemoHIM had higher activity ($p < 0.01$ and $p < 0.04$ in E:T ratio = 50:1 and 100:1, respectively) than the aged mice fed with water only, although it did not reach the young adult values. Because the NK cells are activated by cytokines such as IFN-γ, this result is in accord with the enhanced IFN-γ production from the ConA-stimulated lymphocytes in the HemoHIM-fed aged mice (Fig. 1B).

HemoHIM restores the shifted Th cell response in the aged mice

The process of aging appears to exhibit a dynamic type 2-to-type 1-to type 2 cytokine profile (Shearer, 1997). In the T cell population of aging, there is a dysregulation between the Th1 and Th2 subsets, resulting in a predominant production of Th2 cytokines (Cakman *et al.*, 1996). Because the prototypic Th1 and Th2 cytokines are IFN-γ and IL-4, respectively (Mosmann and Coffiman, 1989; Romagnani, 1997), the IFN-γ and IL-4 levels produced by the antigen (OVA)-stimulated lymphocytes were measured. As expected, the IFN-γ production was markedly decreased in the aged mice when compared with that of the young adult mice (Fig. 3), but its production in the aged mice fed with HemoHIM was significantly restored ($p < 0.03$). In contrast, the IL-4 production was significantly increased (Fig. 3) in the aged mice only fed with water. However, the IL-4 production was normalized in the aged mice fed with HemoHIM.

To directly investigate the Th1/Th2 response *in vivo*, the antigen (DNP-KLH)-specific immunoglobulin level was measured. Th1 cells tend to provide the IgG2a class of antibody, while the Th2 cells are more efficient in providing help for the B cells and the IgE and IgG1 classes of antibodies (Mosmann and Coffiman, 1989; Constant and Bottomly, 1997). As shown in Fig. 4, the IgG2a production was reduced and the IgG1 was increased in the aged mice fed with water, when compared with the young adult mice. However, in the aged mice fed with HemoHIM, the IgG2a production as well as IgG1 was normalized.

These results show that the Th cell response was shifted to a Th2 immune response in the aged mice and HemoHIM supplementation significantly restored the balance of the Th1/Th2 response.

HemoHIM restores the IL-12p70 production in aged mice

Next the serum levels of the total IL-12 and its biologically active form, IL-12p70, were measured, because the IL-12p70 secreted from antigen-presenting cells is known to induce differentiation of Th cells to Th1 cells (Gately *et al.*, 1998). Therefore the total IL-12 and IL-12p70 were measured in the serum of DNP-KLH
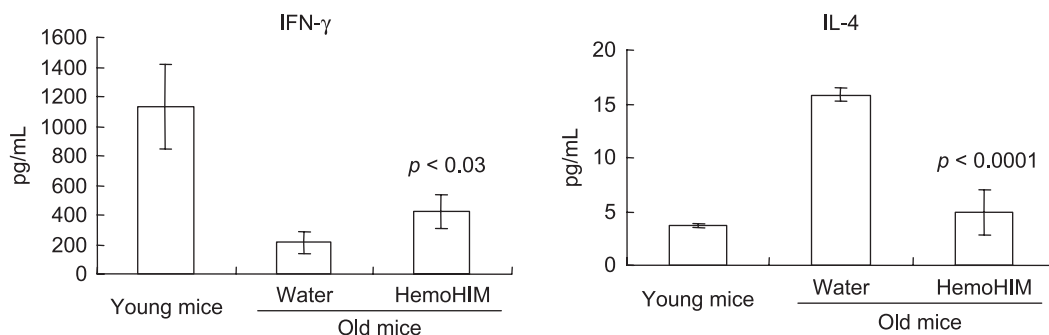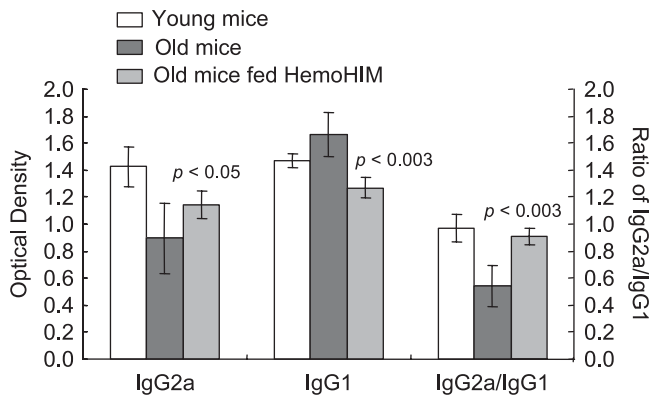

Figure 3. Balanced restoration of the immune response to antigen by HemoHIM in the aged mice. Unfractioned spleen lymphocytes (5 × 10^6 cells/well) obtained from the young adult or aged mice were stimulated with OVA (500 µg/well) for 72 h culture. The levels of IFN-γ and IL-4 in the culture supernatants were measured by an indirect ELISA. There were eight mice in each group. Data show the mean ± SD. * p values mean a significant difference compared with the old mice control.

Figure 4. Balanced restoration of the antigen-specific antibody production by HemoHIM in the aged mice. Mice were intraperitoneally immunized with DNP-KLH (100 µg/mouse). The mice were bled from retro-orbital veins 1 week after immunization. The serum anti-DNP IgG2a and IgG1 concentrations were measured by indirect ELISA. There were eight mice in each group. Data show the mean ± SD. * p values mean a significant difference compared with the old mice control.

sensitized mice. However, the serum IL-12p70 level was lower than the detection limit (20 pg/mL) of the ELISA system. As shown in Fig. 5A, aged mice produced less total IL-12 when compared with the young adult mice ($p < 0.001$), but HemoHIM restored total IL-12 production in the aged mice.

To measure the ability of antigen presenting cells to produce the bioactive form of IL-12 (IL-12p70), spleen cells of the DNP-KLH sensitized mice were re-stimulated with DNP-KLH *in vitro* and the secretion level of IL-12p70 into a cultured medium was measured. As shown in Fig. 5B, a much smaller amount of IL-12p70 was secreted by spleen cells from the aged mice when compared with the young adult mice. However, the spleen cells from the aged mice fed with HemoHIM secreted a markedly increased amount of IL-12p70 when compared with the control aged mice, to near the level of the young adults.

DISCUSSION

The interest in age-related biological change is growing fast according to a rapid increase of the elderly population. Age-related changes show an increased incidence of diseases related to an impaired immune response, such as opportunistic infections, neoplasias and autoimmune diseases.

One of the most important biological effects of aging is an increase in the rate of free radical formation, accompanied by oxidative damage to cellular structure and function. The age-related immune changes may be linked to an age-related decline in antioxidant competence and, more specifically, to a progressive oxidation of glutathione and other thiolic compounds in the tissues of vertebrates and invertebrates (Miquel and Weber, 1990). It is expected that dietary administration of antioxidants that have been shown to prevent excessive oxidation and slow down senescent change, in particular with regard to immune-decline, would restore immune functions deficient in the aged. Although vitamin E seems useful for an immunological restoration in young adult mice but not in aged mice (Bendich, 1996; Wakikawa *et al.*, 1999), it is still promising to use antioxidants as antiaging agents.

HemoHIM is designed to protect self-renewal tissues and to promote the recovery of the immune system against oxidative stress such as irradiation. In our previous studies, HemoHIM was tested for its efficacy as a radioprotective and immunomodulatory agent (Park *et al.*, 2005; Jo *et al.*, 2005; Park *et al.*, 2006). This study investigated the effect of the herb composition, HemoHIM, on the restoration of immune functions which had been impaired in aged mice.

Aging of the immune system is associated with dramatic reductions in immune responsiveness as well as functional dysregulation (Solana *et al.*, 1991; Miller, 1996). The decline of immunity occurs mainly in the T cell-dependent immune functions (Hirokawa *et al.*, 1994; Globerson, 1995; Pawelec, 1995). The most dramatic change with age is reported to occur within the T-cell compartment where there is a declined function of both the CD4$^+$ and CD8$^+$ cells. *In vitro* studies on the effects of age on cell-mediated immunity have mainly focused on the response of T lymphocytes to mitogenic stimuli. The age-related decline of immunity is manifested mainly by impairment in the lymphoproliferative response (Solana *et al.*, 1991; Song *et al.*, 1993) and cytokine secretion (Rink *et al.*, 1998) to mitogenic stimuli. Cytokines are a key component in the regulatory communication among immune cells; they are responsible for the differentiation, proliferation and survival of the lymphoid cells and play an important role in immune
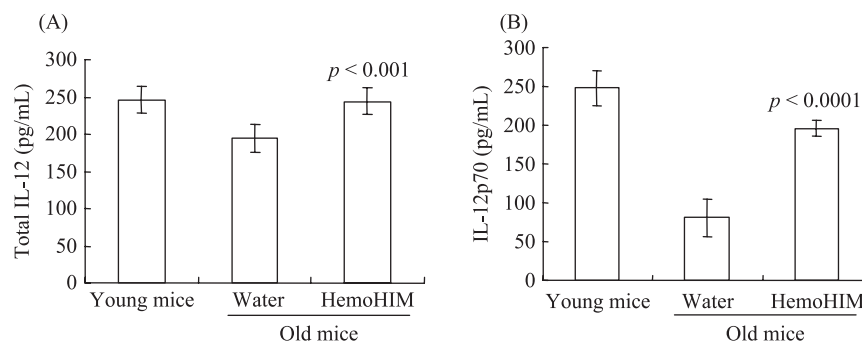

Figure 5. Restorative effects of HemoHIM on the total IL-12 and IL-12p70 secretion in the aged mice. Mice were immunized with DNP-KLH (100 µg/mouse) 7 days in advance of preparing the spleen cells. (A) The mice were bled from the retro-orbital veins and then the total IL-12 in the serum was measured by indirect ELISA. (B) The spleen cells from these immunized mice were re-stimulated by DNP-KLH (200 µg/well) *in vitro* for 18 h. The levels of IL-12p70 in the culture supernatants were measured by indirect ELISA. There were eight mice in each group. Data show the mean ± SD. * p values mean a significant difference compared with the old mice control.

responses and inflammation. Previous studies have shown decreased secretion of IFN-γ and IL-2 in the aged, which are known to be correlated to a change of differentiation and proliferation of the lymphocytes (Rink *et al.*, 1998). In our data, the proliferative response of spleen lymphocytes to ConA stimuli was significantly decreased in the aged mice. However, HemoHIM recovered the response of lymphocytes to ConA. The data also showed that lymphocytes from the aged mice produced significantly less IFN-γ and IL-2 in response to ConA. HemoHIM recovered the IFN-γ and IL-2 production of ConA-stimulated lymphocytes from the aged mice. These data suggest the ability of HemoHIM to overcome immunosenescence of lymphocytes in aged mice.

An age-associated decline in NK activity has also been found by many investigators (Hirokawa *et al.*, 1994; Utsuyama *et al.*, 2001; Plackett *et al.*, 2004; Mocchegiani and Malavolta, 2004). The number of NK cells increases with ageing (Sansoni *et al.*, 1993; Xu *et al.*, 1993), but its cytotoxic activity does not (Franceschi *et al.*, 1995). In our data, NK cell activity was recovered in the aged mice fed with HemoHIM. This result is in accord with the result that HemoHIM enhanced the IFN-γ production of lymphocytes from aged mice, since NK cells are also known to produce IFN-γ and to be subject to the influence of IFN-γ (Trinchieri, 1989).

The balance of the Th1 and Th2 related cytokines is important in the protection against both intra- and extra-cellular infection. However, murine models of aging have demonstrated that there was an age-associated dysregulation in the cytokine production pattern, as evidenced by a consistently decreased production of IFN-γ and IL-2 (Shearer, 1997) and a generally increased production of IL-4 (Hobbs *et al.*, 1993; Albright *et al.*, 1995) and IL-10 (Cakman *et al.*, 1996). Our results showed a Th1/Th2 imbalance in the aged mice, in that IL-4 as well as IgG1 secretion, mediated by Th2 cells, was increased, whereas the IFN-γ and IgG2a production were decreased. The administration of HemoHIM restored the Th1/Th2 balance in the aged mice.

IL-12 is known to enhance the IFN-γ secretion level by Th cells and the lytic activity of the NK/LAK cells (Chan *et al.*, 1991; Trinchieri, 1995). Several studies in humans (Wu *et al.*, 1993, Manetti *et al.*, 1994) and in mice (Seder *et al.*, 1993, Trinchieri, 1994) have assigned a role to IL-12 as the promoter of Th1 cell generation, acting antagonistically with IL-4, the major promoter of Th2 responses. The early preference expressed in the immune response depends on the balance between IL-12 and IL-4. In this study, it is suggested that, as a likely mechanism, the depression of the Th1-like immune response in the aged mice was due to a lesser IL-12 secretion. In this study, the secretion of IL-12p70 was very low in the aged mice, but the HemoHIM administration recovered the IL-12p70 secretion. This result implies that a factor in the Th1/Th2 imbalance in the aged mice might be lower IL-12p70 secretion and the restoration of the Th1/Th2 balance in the aged mice fed with HemoHIM might be due to the recovery of IL-12p70 secretion.

In conclusion, despite insufficient information to define the optimum immune function in the aged mice, this study confirmed that HemoHIM improved or postponed the decline in the immune responsiveness that occurs with aging in mice. The results of the current study suggest that HemoHIM might be a useful public health measure aimed at restoring the immune functions of elderly people.

Acknowledgements

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