

# Suppressive Effect of Hochu-Ekki-To on Collagen Induced Arthritis in DBA1J Mice

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**ABSTRACT. Objective.** To investigate the effect of hochu-ekki-to (HET) decoction on the development of collagen-induced arthritis (CIA) in mice.

**Methods.** CIA was induced in male DBA/1J mice by immunization with 2 injections of bovine type II collagen (CII). HET was orally administered at different doses and with different schedules. The incidence of arthritis, arthritis index, levels of anti-CII antibody, interleukin 6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and lymphocyte subsets were examined.

**Results.** HET caused suppression of CIA development in a dose dependent fashion and exerted a suppressive effect on CIA when administered from the first CII immunization or from the onset of CIA, but not when administered for 2 weeks before CII immunization. HET inhibited the production of specific anti-CII antibody, IL-6, and TNF- $\alpha$ , and tended to normalize the proportions of cells in lymphocyte subsets.

**Conclusion.** HET suppresses the development of CIA, and HET redistributes the population of lymphocytes in lymph node and blood and inhibits IL-6 and TNF- $\alpha$  secretion in CIA mice. (J Rheumatol 2002;29:1601-8)

*Key Indexing Terms:*

HOCHU-EKKI-TO (BU-ZHONG-YI-QI-TANG)  
IMMUNOMODULATION

COLLAGEN-INDUCED ARTHRITIS  
ALTERNATIVE MEDICINE

Collagen-induced arthritis (CIA) has been considered a useful animal model for studying pathological mechanisms and therapeutic agents of rheumatoid arthritis (RA). This experimental model shows many features that mimic RA in humans. For example, RA, synovitis, and erosion of cartilage and bone are hallmarks of CIA; susceptibility to both RA and CIA is linked to the expression of specific MHC class II molecules.

Although CIA is not identical to RA, the features of CIA clearly indicate that an autoimmune reaction to a cartilage component can lead to chronic, destructive polyarthritis. Although CIA can be induced in susceptible strains of rodents and primates by immunization with heterologous type II collagen (CII)<sup>1-3</sup>, it is the autoreactive component of the immune response that leads to disease<sup>4</sup>. CIA development has been shown to involve both cellular and humoral immunity to CII, and passive transfer of T lymphocytes sensitized with CII<sup>5</sup> or transfer of CII reactive sera<sup>6</sup> can also induce the disease in DBA/1 mice.

Many kinds of Chinese traditional herbal medicines (Kampo formulas) have been found to be clinically effective for RA treatment. These formulas usually consist of several medicinal plants. They are thought to exert antiinflammatory and immune regulatory effects and to be effective for treating chronic diseases<sup>7-9</sup>. Known in Japan as hochu-ekki-to (HET) and elsewhere by its Chinese name, bu-zhong-yi-qi-tang, HET is composed of 10 species of medicinal plants and is used for chronic diseases or weakness after illness<sup>10</sup>. HET has been widely used to treat patients with certain immune related diseases. Recent studies have shown that HET formula also exhibits immunopharmacological activities such as increased protection against tumors<sup>11,12</sup>, and protection against bacterial<sup>13</sup> and viral infection<sup>14</sup>. Kaneko, *et al* reported that HET also exerts anti-allergic activity by regulating some cytokines<sup>15</sup>. Our previous report showed that HET caused a reduction of soluble CD23, a marker of

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activated B cells in a patient with RA, as well as improvement in joint symptoms<sup>16</sup>. However, the mode of action of HET on RA and the scientific basis for using this Kampo medication for RA treatment have not been investigated.

We examined the effects of using HET for treatment of RA. For this purpose, the CIA model in DBA/1J mice was used to evaluate the effects of HET on the immune response to CII.

## MATERIALS AND METHODS

**Animals.** Eight-week-old male DBA/1J mice purchased from Sankyo Laboratories, Tokyo, Japan, were used. The mice were kept in a temperature controlled room (at 23°C) with a 12 h light/dark cycle, housed in polystyrene cages and given standard rodent chow and water ad libitum. Mice were randomly separated into normal, non-immunized (Nor), untreated CII-immunized (CONT), and CII-immunized HET-treated (HET) groups.

**Hochu-ekki-to preparation.** In this study, HET was prepared by mixing the 10 herbs (purchased from Tochimoto Co. Ltd., Osaka, Japan), all in dried, crude form and preparing an extract from them. One hundred and twenty grams of the mixed material was mixed with 500 ml of distilled water and heated for 60 min at 100°C. The extract was filtered, and the residue was extracted once more according to the same procedure. Finally, all extract fractions were pooled and kept at 4°C for 24 h, when the insoluble substances were removed by centrifugation at 12,000 rpm for 30 min. The supernatant was concentrated by rotary evaporation, and lyophilized to form a dried powder. The freeze dried powder was stored at -20°C until use.

**Analysis of 3D-HPLC finger-print of hochu-ekki-to.** HET (2.5 g, Tsumura Co., Ltd Tokyo, Japan) was filtered and then submitted for high performance liquid chromatography (HPLC) analysis. HPLC equipment was controlled with a SLC-10A (Shimazu, Kyoto, Japan) using a TSK-GEL ODS-80TS column (4.6 × 250 mm), eluting with solvents (A) 0.05 M

AcONH<sub>4</sub> (pH3.6) and (B) CH<sub>3</sub>CN. A linear gradient of 100% A and 0% B changing over 60 min to 0% A and 100% B was used. The flow rate was controlled with LC 10AD pump as 1.0 ml/min. The eluate from the column was monitored and the 3D data was processed by SPD-M10A diode array detector. The profile is shown in Figure 1. HET is a crude drug composed of several ingredients. To demonstrate the characteristics of HET in this study, analysis of 3D-HPLC was carried out.

**Introduction of collagen-induced arthritis and hochu-ekki-to treatment.** The immunization reagent was freshly prepared as follows: bovine CII (K42) (Sankyo Laboratories), stored at -20°C until use, was thawed and dissolved in 0.1 M acetic acid at 2.0 mg/ml. The solution was emulsified in an equal volume of complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI, USA). Collagen-induced arthritis (CIA) was induced in DBA/1J mice by intradermal injection of 0.2 ml of emulsion into the base of the tail. Three weeks later, a second immunization, the booster, was carried out in the same manner<sup>1</sup>. The day of the second immunization was considered as Day 0. From the day of the first immunization until the end of the experiment, HET-treated and CONT mice were administered HET or vehicle only (water) daily using a gastro tube. HET was administered at 0.1, 0.5, or 2.5 g powder/kg/day. HET treatment was also carried out with some different time courses.

**Clinical assessment of arthritis.** Clinical arthritic symptoms were evaluated every 2 days after the booster. Joint inflammation was evaluated with a visual scoring system for each paw on a scale of 0–3 points<sup>8</sup>: 0 = no change, 1 = swelling and erythema of the digit in 1 joint; 2 = mild swelling of more than 1 joint and erythema of the limb; and 3 = gross swelling and erythema of the limb. The scores of the 4 limbs were summed, and thus arthritis severity could range from 0 to 12 for each mouse. A mouse was considered to have arthritis if it had a score of 1 or more. The mean arthritis scores represent the severity in only the animals that developed CIA in each group. The incidence and day of onset of arthritis were also recorded. The day of onset was considered as the day of first appearance of joint inflammation.

**Quantification of anti-CII antibody in sera.** Sera were collected on Day 21 after the booster and kept at -20°C until use. For the quantification of anti-

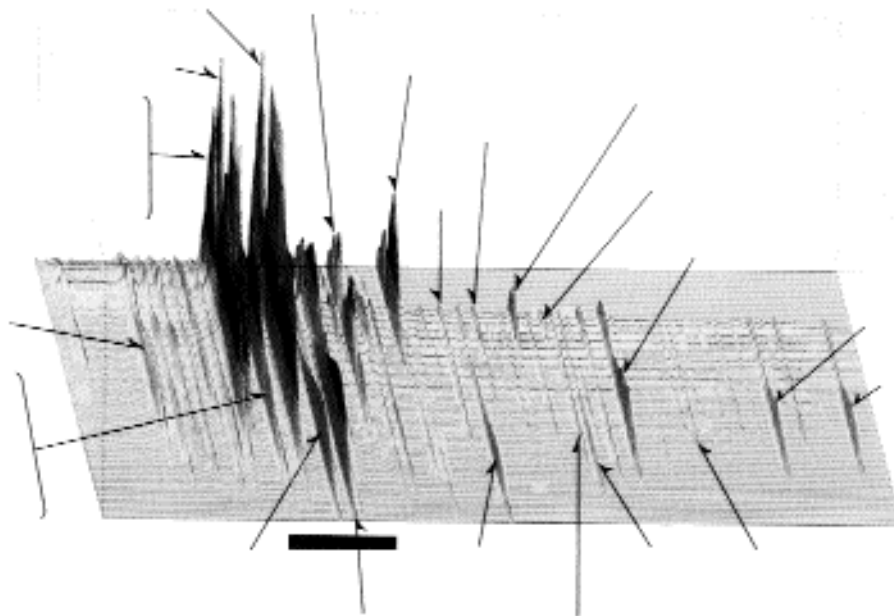


Figure 1. The 3 dimensional high performance liquid chromatography profile of hochu-ekki-to.

CII reactive autoantibodies in sera, an ELISA technique was used. Ninety-six-well micro-ELISA plates were coated with 100  $\mu$ l/well of soluble bovine CII at a concentration of 10  $\mu$ g/ml in phosphate buffered saline (PBS) by incubation overnight at 4°C. Plates then were washed 3 times with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-Tween) and blocked by incubation with 100  $\mu$ l/well of 3% bovine serum albumin in PBS overnight at 4°C. Plates were then thoroughly washed 3 times with PBS-Tween, and incubated with serum diluted 1:1000 in PBS for 2 h at room temperature. After 3 washes with PBS-Tween, alkaline phosphate-conjugated goat anti-mouse IgG antibody (BioRad Laboratories, Hercules, CA, USA) diluted 1:3000 in PBS was added to each well. After 2 h of incubation at room temperature, plates were washed thoroughly in PBS-Tween and then the bound enzyme was quantified with a paranitrophenol-containing substrate buffer. The absorbance was determined by using a microplate reader (Model 450, BioRad) at a wavelength of 405 nm and a reference wavelength of 490 nm. Each sample was tested in triplicate. The titer of specific anti-CII IgG in each group was expressed as the mean  $\pm$  SE of optical density values.

*Quantification of circulating interleukin 6 and tumor necrosis factor- $\alpha$  cytokines in sera.* For this experiment, CIA was induced in mice as described above. HET (0.5 g/kg/day) was orally administered from first immunization until the end of the experiment. Sera were collected on Day 21 after the booster and stored as described above. Concentrations of interleukin 6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in sera derived from non-immunized mice (Nor), HET-treated mice, and untreated (CONT) mice were quantified using mouse IL-6 and TNF- $\alpha$  ELISA kits (Cosmo bio Co., Ltd, Tokyo, Japan). Each sample was assayed in duplicate to determine the average value as recommended by the supplier, and the data were expressed as mean  $\pm$  SE in each group.

*Flow cytometric analysis of lymph cell subsets in the lymph nodes and blood.* Mice were sacrificed on Day 21 after the second immunization based on the results of previous study<sup>17</sup>. Heparinized blood samples were collected by heart puncture and peripheral blood mononuclear cells were isolated by gradient centrifugation using Lympho Prep (Nyegaard, Oslo, Norway). Axillary and inguinal lymph nodes were also harvested. Lymph tissues were dispersed in PBS using a fine stainless steel mesh. Then the lymphocyte suspension was collected and treated for 5 min in hemolytic buffer (NaCl 138 mM, KHCO<sub>3</sub> 0.01 M, 2NaEDTA 0.1 mM, pH 7.4) to remove erythrocytes, and the lymphocytes were washed twice in PBS and collected by centrifugation at 1500 rpm for 15 min. Then the cells were resuspended in PBS to form a suspension of  $1 \times 10^6$  cells/ml. One milliliter of cell suspension at  $1 \times 10^6$  cells/ml was used for flow cytometric analysis to detect lymphocyte subsets by a double staining technique. To analyze B220+ (B lymphocyte), TCD3+CD4+ and TCD3+CD8+ lymphocyte subsets,  $1 \times 10^6$  cells were stained by incubation with 10  $\mu$ l of fluorescein isothiocyanate labeled anti-mouse CD3 monoclonal antibody and 10  $\mu$ l of phycoerythrin labeled anti-mouse B220, CD4, or CD8 antibody (Immunotech, Marseille, France) respectively, at room temperature for 45 min in the dark. After incubation, the suspension was analyzed using a flow cytometer (Epics XL, Beckman Coulter, France).

*Statistical analysis.* The data were analyzed by chi-square test, or Mann-Whitney U test using Stview v 4.5 software.  $P < 0.05$  was considered significant.

## RESULTS

*Suppressive effect of HET on the development of CIA.* We administered HET daily to the mice from the day of the first CII injection until the end of the experiment in order to evaluate the effect of HET on the development of CIA. In both the CONT and HET groups, no symptoms were observed before the second CII injection.

We first examined the incidence of CIA development in CII immunized mice. Table 1 shows that HET treatment

resulted in a significant reduction in the incidence of CIA. At Day 20 after booster, only 66.7% (10/15) of mice in the HET 0.1 g/kg (HET 0.1) treated group, and 57.1% (24/42) of mice in the HET 0.5 g/kg (HET 0.5) treated group developed CIA, in contrast to 92.8% (26/28) in the CONT group.

We observed a delay of the onset of CIA symptoms by HET treatment (Table 2). The first symptoms of CIA (onset) appeared around Day 6 after the second CII injection in the CONT group. HET 0.5 g/kg treatment from the day of the first CII injection significantly delayed the onset of CIA, until Day 10 after the booster (\*  $p < 0.05$ , by Mann-Whitney U test). However, at a low dose of HET (0.1 g/kg), no delay of onset was seen.

*Dose dependent effect of HET on the progression of CIA.* We observed dose dependent suppression of the clinical progression of CIA by HET treatment (Figure 2). The severity of CIA was expressed as the arthritis index score only in animals that developed CIA. In this experiment, HET treatment was performed at 0.1, 0.5, and 2.5 g/kg. In the CONT group, the arthritis severity was significantly more serious than that in the HET-treated groups at all indicated time points. Arthritic inflammation in the CONT group was serious, with swelling of the entire paw in most mice. In the HET-treated groups fewer mice had swelling of the entire paw. Figure 2 also shows that the suppressive effect of HET on the progression of CIA was dose dependent. HET treatment at 0.5 g/kg (HET 0.5) and 2.5 g/kg (HET 2.5) was clearly more effective than that at 0.1 g/kg (HET 0.1). Moreover, no significant difference was found between 0.5 and 2.5 g/kg of HET.

*Antiarthritic effect of HET with different schedule of treatment.* We next analyzed the effects of the time course of HET treatment on the development and severity of arthritis. In this experiment, CII immunized mice were orally administered HET 0.5 g/kg daily in the following protocols: only for 2 weeks before first CII immunization; from the first CII immunization; and from the day of onset (considered as the day on which arthritis was established). Figure 3 shows that HET pretreatment for 2 weeks before CII challenge (HET-pre) had no effect on the development of CIA. However, the antiarthritic effect of HET was exhibited when treatment was started on the day of the first CII immunization (HET-1st) and when treatment was started in mice with established arthritis (HET-est).

*Change in the level of specific anti-CII IgG antibody.* To determine the effect of HET on the humoral immune response in CIA, we measured the levels of specific anti-CII IgG antibody in sera by ELISA. The level of specific anti-CII IgG antibody was significantly reduced from 0.91 in untreated (CONT) mice to 0.558 in HET-treated mice (Figure 4).

Antibody production is in part regulated by polyclonal B cell activation<sup>18-20</sup>. Therefore, the inhibition of anti-CII IgG antibody production may be related to inhibition of B cell

**Table 1.** Effect of HET treatment on the incidence of CIA onset and development. DBA/1J mice developed CIA after 2 interval challenges of bovine CII. Hochu-ekki-to 0.1 g/kg (HET 0.1), 0.5 g/kg (HET 0.5), 2.5 g/kg (HET 2.5), or vehicle only (CONT) was administered orally from the days of first CII immunization. Data at indicated time points are mean  $\pm$  SE.

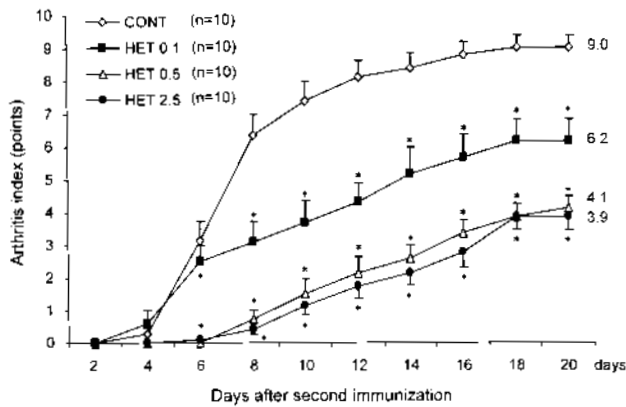
Group	No. of Mice	No. of Mice with CIA 20 Days after Booster (%)	Onset, Days $\pm$ SE
CONT	28	26 (92.9)	6.0 $\pm$ 0.31 (n = 26)
HET 0.1	15	10 (66.7)*	5.8 $\pm$ 0.68 (n = 10)
HET 0.5	42	24 (57.1)**	10.0 $\pm$ 0.51 (n = 24)*
HET 2.5	20	13 (65.0)**	9.0 $\pm$ 0.65 (n = 13)*

\*  $p < 0.05$ ; \*\*  $p < 0.01$  vs CONT by chi-square test (for CIA incidence) or by Mann Whitney U test (for CIA onset).

**Table 2.** Serum concentrations of IL-6 and TNF- $\alpha$ . Serum concentration (pg/ml) of interleukin 6 (IL-6), n = 12 in each group, and tumor necrosis factor alpha (TNF- $\alpha$ ), n = 16 in each group, in CIA untreated mice (CONT) and HET 0.5 g/kg treated mice (HET 0.5). Results are mean  $\pm$  SE.

	CONT	HET 0.5	NOR
IL-6	45.07 $\pm$ 13.07	8.45 $\pm$ 2.58*	No test
TNF- $\alpha$	16.73 $\pm$ 1.56	12.39 $\pm$ 1.04*	5.2 $\pm$ 0.33

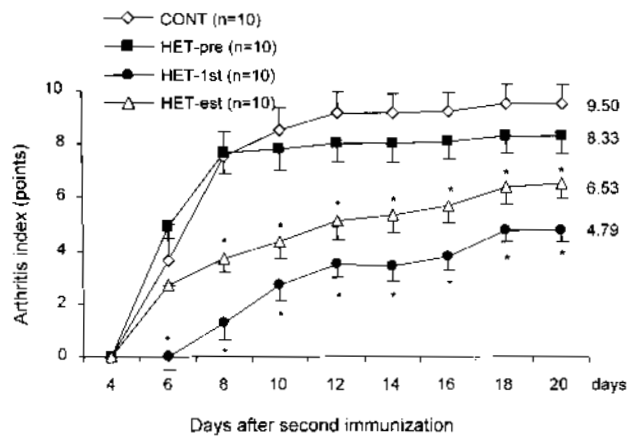
\*  $p < 0.05$  in Mann-Whitney's test.



**Figure 2.** Suppressive effect of HET treatment on the progression of CIA. CIA was induced in DBA/1J mice by 2 injections of CII. Mice were orally treated from the day of the first injection with HET 0.1 g/kg (HET 0.1), 0.5 g/kg (HET 0.5), and 2.5 g/kg (HET 2.5) or untreated (CONT). The arthritis index in each group is presented as mean  $\pm$  SE. \* $p < 0.05$  vs CONT by Mann-Whitney U test.

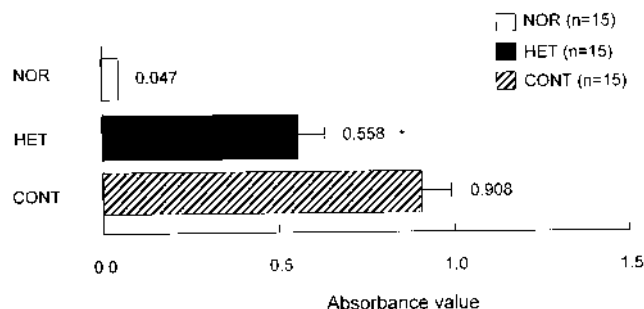
activation, and the mechanism of the therapeutic effect of HET on CIA might involve the suppression of B cell activation.

**Change in serum IL-6 and TNF- $\alpha$  levels.** Next we examined the effects of HET treatment on the serum concentrations of cytokines IL-6 and TNF- $\alpha$  (Table 1). In the CONT group, the serum levels of IL-6 and TNF- $\alpha$  dramatically increased. HET treatment significantly reduced the IL-6 serum level from 45.07 pg/ml in controls to 8.45 pg/ml in HET-treated



**Figure 3.** Effect of HET treatment schedule on the severity of CIA. CIA was induced in DBA/1J mice by 2 injections of CII, and the mice were orally administered by HET 0.5 g/kg or vehicle only. HET-pre: HET-treated only for 2 weeks before first CII-injection; HET-1st: HET-treated from first CII injection; HET-est: HET-treated from day of CIA onset. Arthritis index in each group is presented as mean  $\pm$  SE. \* $p < 0.05$  vs CONT by Mann-Whitney U test.

mice. HET also reduced TNF- $\alpha$  serum levels. HET treatment also significantly decreased the concentration of circulating TNF- $\alpha$  from 16.73 pg/ml in untreated (CONT) mice to 12.39 pg/ml in HET-treated mice. These results suggest that the modulation of IL-6 and TNF- $\alpha$  synthesis might contribute to the therapeutic effect of HET.



**Figure 4.** Suppressive effect of HET treatment on serum level of specific anti-CII antibody. Mice were injected with CII twice to induce CIA and treated with HET or water only. Blood samples were collected on Day 21 after booster. Specific anti-CII IgG Ab concentrations are presented as mean absorbance values  $\pm$  SE. Nor: normal nonimmunized mice; HET: hochu-ekki-to 0.5 g/kg treated mice; CONT: untreated mice. \* $p < 0.005$  vs CONT by Mann-Whitney U test.

**Lymphocyte subset partition change in lymphatic tissues.** Figure 5 shows the lymphocyte subset partitions in blood and lymph nodes derived from Nor, control, and HET mice. In CONT mice, B cell (B220 positive cell) of both lymph node and blood subpopulations increased markedly compared with those from normal mice (Nor), from 28% (in the Nor group) to 53% in the lymph nodes and from 32% to 50% in blood. The increase in the B cell subpopulation was significantly inhibited in the HET-treated group; the proportion of B cells was only 36% (in lymph node) and 40% (in blood) compared with 53 and 50% in CONT mice.

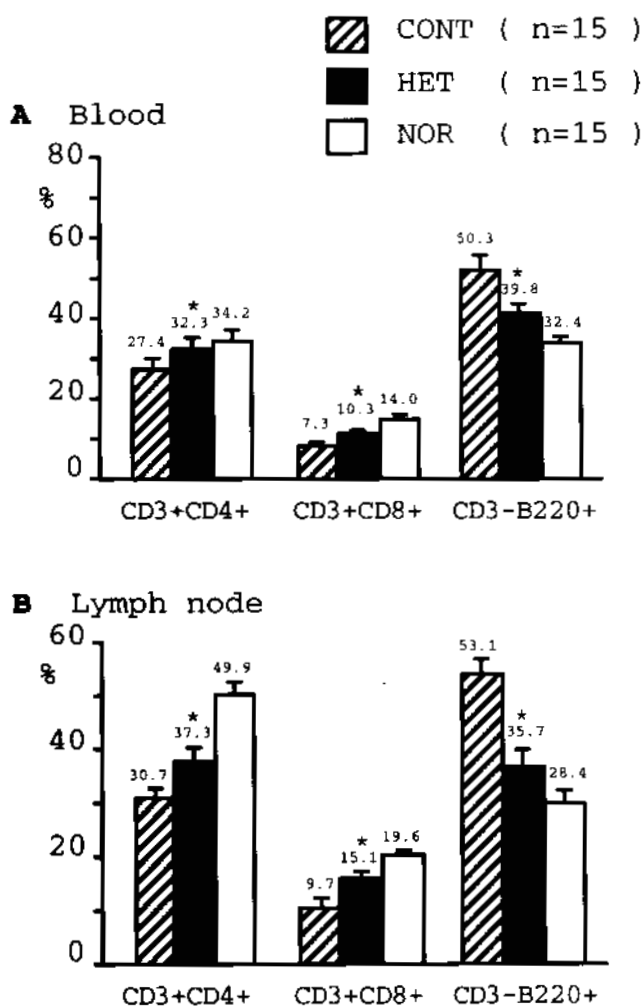
We found significant reductions of not only CD8+ but also CD3+ and CD4+ T cells in both the lymph nodes and blood of mice with CIA (both untreated and HET-treated mice) compared with the Nor group. Although they were still significantly lower than in normal mice (Nor), the levels of CD3+, CD4+ and CD8+ T lymphocytes in HET-treated mice were much closer to the normal levels in both lymph nodes and blood.

The absolute count of lymphocytes was also calculated in blood and lymph nodes (Table 3). In lymph nodes, changes in the subpopulation based on absolute cell count were similar to changes in cell percentages. In blood, the count of each population increased in untreated CIA mice. B cells significantly decreased by the treatment of HET.

## DISCUSSION

HET is a herbal formula containing 10 species of medicinal plants. Traditionally, a water decoction of this herbal formula is commonly used in Oriental medicine to treat patients with chronic diseases including RA or weakness after illness<sup>10</sup>.

Recently, we reported the successful use of HET to treat a patient with RA<sup>16</sup>. In experimental animal models, HET exhibits immunopharmacological activities such as



**Figure 5.** Lymphocyte subset partitions in blood (A) and lymph nodes (B). Mice were injected with CII twice to induce CIA and orally treated from the day of the first injection with HET or vehicle only. Three weeks after booster, lymphocytes were isolated from spleen, blood, and lymph nodes and analyzed by flowcytometer. The levels of CD3-B220+ cells increased and the levels of CD3+CD4+ and CD3+CD8+ cells decreased in CONT mice compared with Nor mice. The levels of CD3-B220+ cells and CD3+CD4+, CD3+CD8+ cells in HET-treated mice were much closer to the normal levels. \* $p < 0.005$  vs CONT by Mann-Whitney U test.

enhancement of protection against tumors<sup>11,12</sup>, protection against infection by bacteria<sup>3</sup> and viruses<sup>14</sup>, and anti-allergic activity<sup>15</sup>. Therefore, we were interested in whether HET treatment improves RA, and how HET works. This is the first report about the effect of HET on CIA, an animal model of RA.

We previously reported a case of RA effectively treated with HET, with improvement of immune status as well as joint symptoms<sup>16</sup>. These effects may have resulted from modulation of the immune response to CII. Although we do not yet completely understand the mechanism of the anti-

**Table 3.** Total cell counts ( $1 \times 10^6$  cells) of CD4 and CD8 T cells and B cells. Mice were orally administered water only (in CONT group) or HET 0.5 g/kg/day from first injection. Three weeks after booster, lymphocytes were collected from axillary and inguinal lymph nodes and blood. Cell counts of CD4, CD8 T and B cell subsets in regional lymph nodes and blood samples were calculated based on the ratio of these cell subsets obtained in the flow cytometric analysis, and the total cell counts of lymphocytes were simultaneously calculated with a hemocytometer. Mean  $\pm$  SD of stained cells in the regional lymph node (15 mice per group).

Cell Type	Group	Lymph Node	Blood
Total	Normal	260.7 $\pm$ 97.7	5.57 $\pm$ 1.32
	CONT	498.7 $\pm$ 105.9	13.89 $\pm$ 2.32
	HET	434.7 $\pm$ 171.2	12.75 $\pm$ 3.35
B cell	Normal	74 $\pm$ 27.8	1.81 $\pm$ 0.43
	CONT	264 $\pm$ 56.2	6.99 $\pm$ 1.17
	HET	155.2 $\pm$ 61.1*	5.08 $\pm$ 1.33*
CD4 T cell	Normal	130.1 $\pm$ 48.8	1.9 $\pm$ 0.45
	CONT	153.1 $\pm$ 32.5	3.80 $\pm$ 0.64
	HET	162 $\pm$ 63.9	4.12 $\pm$ 1.08
CD8 T cell	Normal	51.1 $\pm$ 19.2	0.78 $\pm$ 0.18
	CONT	48.4 $\pm$ 10.3	1.01 $\pm$ 0.36
	HET	65.6 $\pm$ 25.9*	1.31 $\pm$ 0.34

\*  $p < 0.05$  vs CONT by Mann-Whitney's U test.

RA activity of HET, the laboratory findings help us to explain in part these activities.

The serum level of specific anti-CII IgG was reduced in the HET-treated group, indicating that HET treatment can inhibit the production of IgG specific for CII. This observation is interesting, because the production of anti-CII antibodies is thought to be important for the induction of arthritis, since the disease can be transferred by injection of antibodies specific for CI<sup>6</sup>, and anti-CII antibodies cause deposition of immune complexes in the synovium or cartilage<sup>21</sup>.

Additionally, it is known that the production of anti-CII antibodies is associated with B cell activation, as well as cellular immunity. In CIA, blocking B cell activation by treatment with anti-CD40 ligand leads to protection against the disease and a total block of the antibody response<sup>20</sup>. Thus, B cell activation in the introduction phase plays a critical role in the development of CIA. We observed that oral administration of HET reduced the serum concentration of anti-CII antibody. This reduction seems likely the result of suppression of B cell activation, and this effect might contribute to the suppression of CIA development.

We previously reported that HET treatment decreased the serum concentration of soluble CD23, a marker of B cell activation<sup>16</sup>. These immunomodulatory effects are not like those of nonsteroidal antiinflammatory drugs (NSAID) because NSAID do not suppress the production of autoantibodies<sup>22</sup> secreted from peripheral blood mononuclear cells. However, although the present study showed that HET treatment reduced the percentage of B cells in lymph nodes and blood, it did not examine whether the population of acti-

vated B cells decreased during HET treatment. To answer this question, further studies of the population of activated B cells such as CD86 positive cells will be required.

IL-6, a proinflammatory cytokine, is known to be involved in the inflammation and pathology of CIA. Recent studies showed the essential role of IL-6 produced after CII immunization in both the humoral and cellular immunity to CII. Overproduction of IL-6 in serum and synovial tissue has been reported in mice with CIA<sup>23,24</sup>, and the development of CIA can be reduced by suppressing IL-6 signal transduction. These findings suggested that blockage of IL-6 or IL-6 receptor might be beneficial for the treatment of RA<sup>24-26</sup>.

In the present study we found that HET treatment started immediately after the first CII immunization markedly reduced the serum level of IL-6, and we think that the inhibitory effect of HET on CIA progression may be partially mediated by modulation of the production of the proinflammatory cytokine IL-6. Since RA is a chronic, immune mediated inflammatory disease, cytokine network disorders may have a close relation with the occurrence and development of RA. TNF- $\alpha$ , as well as IL-6, is also thought to be a key cytokine in this network. High concentrations of TNF- $\alpha$  are often found in blood and synovial fluid from RA patients as well as in synovial tissue of mice with CIA<sup>23</sup>. Treatment of RA patients with anti-TNF- $\alpha$  monoclonal antibody or agents that neutralize TNF- $\alpha$  clearly modified the Th1/Th2 balance<sup>27</sup> and improved the disease condition<sup>28</sup>. TNF- $\alpha$  treatment in the introduction phase suppresses the development of CIA in DBA/1 mice<sup>29</sup>. Our study revealed that HET treatment from the first CII immunization also inhibited the production of TNF- $\alpha$  in serum and reduced CIA development. Thus, HET may also exert an antirheumatic effect by acting as a TNF- $\alpha$  neutralizing agent.

However, there are limitations in attempting to extrapolate implications of CIA experimental observations to human RA: the mechanism is not always identified with human RA although IL-6 or TNF- $\alpha$  plays an essential role in both CIA and human RA. In the CIA model, the activation of T cells and cellular immunity are thought to play a crucial role.

Therefore, to further investigate the effect of HET treatment on the cellular immune response to CII, we studied the partitioning of lymphocyte subsets in blood, lymph node, and spleen by flow cytometry. We observed increased B cells and decreased CD4/8 positive cells in blood and lymph node in accord with several investigators. In HET-treated mice the proportions of CD8+, CD4+ T cells as well as B220+ cells were significantly restored toward normal levels.

These results indicate that the CIA-suppressing effect of HET might be due to modulation of the immune response to CII. In our analysis, the lymph node and peripheral blood

data were not similar to those of spleen, and we still cannot explain this phenomenon. Similarly, previous reports have also shown that spleen data were different from those of peripheral blood<sup>30,31</sup>. Therefore, several other studies are required concerning the subpopulation of spleen cells in CIA-mice.

In addition, we assessed the lymphocyte subpopulation in the thymus (data not shown). In both untreated CIA mice and HET-treated mice, the subpopulation of thymic T cell did not change, suggesting that thymic T cell differentiation might not be associated with the action of HET.

We also assessed the absolute lymphocyte count in blood and lymph nodes. In lymph nodes, changes in the subpopulation based on absolute cell count were similar to changes in cell percentages. In blood, the count of each population increased in untreated CIA mice. B cell count significantly decreased by the treatment of HET.

Taken together, our present findings demonstrated that HET exerts anti-CIA effects via antiinflammatory and immunomodulatory activities. We have not completely clarified the mechanism of this action, but have demonstrated that suppression of the secretion of the proinflammatory cytokines TNF- $\alpha$  and IL-6 and inhibition of B cell activation participate in the mechanism of the antiarthritic activity of HET.

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