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Hemopoietic Formulae Rearranged Leukocyte Subsets and Implication for Use against the Type of Constitution and Infectious Agent for Further Modification to Future

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Abstract

A hemopoietic formula (HF) in traditional Chinese medicine (TCM) usually consists of more than six single herbs. We seek to look into changes of immune-competent cells by two commonly utilized HF, including Shi-Quan-Da-Bu-Tang (SDT) and Bu-Zong-Ye-Qi-Tang (BYT). However, the effects of both formulae on levels of leukocyte subsets are not yet defied. After administration of immno-suppressant to animals, the effects by HFs on the augmentation for subsets of leukocytes, CD positive cell counts of lymphocytes, and the cytokine-producing cells are measured. Our results show that SDT augments the level of lymphocytes, while BYT increases the level of granulocytes. In our clinical study with 15 healthy volunteers, positive cells for CD2, CD11b, CD14, IL-4, IL-1β and IFN-γ in peripheral blood are increased significantly 15 days after the ingestion of HFs. In rodents, compromised host as well as normal animal is administered with cancer chemotherapeutic agent

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(Mytomycin-C). Our observations show that SDT regulates phagocyte activities of the immune system, introducing it against intracellular parasite, and that BYT augments intercellular pathogen through humoral immunity. We discuss the significance and mechanism of HFs on the level of leukocyte subsets in number and function that are considered to be potential indicators for the evaluation of TCM modalities. We also propose an idea that TCM exhibits tonic effects via enhancing the ability to fight against infection and bacteria, as well as turning the multi-drug resistant bacteria to the sensitive ones. Together with these evidences, we suggest an ideal remedy for the compromised to fight host against pathogens. Moreover, we try to modify these hemopoietic formulae to more digestive forms. This modification brings to the significant lifted up for antibody producing cells and anti-oxidative activity for phagocytic cells.

Keywords

Traditional Chinese Medicine, Cancer Chemotherapeutic Agent, Compromised Host, Intracellular Infectious Agent, Intercellular Infectious agent

1. Introduction

In recent years, complementary and alternative medicines (CAM) have achieved more and more attentions since they are able to treat many chronic illnesses, such as fatigue syndrome that plagues the industrialized world. The present team has reported that typical styles of CAM, *i.e.* TCM regulate functions of leukocytes in human immune system [1] [2]. Dietary and hemopoietic formula holds promise as strong inducers of acquired immunity. While the immune system is working against the local infection of pathogens, cytokine and immuno-competent cells react throughout the body in close connection to the brain, the endocrine and immune system [3]. In this study, we hypothesize that HF may influence immuno-competent cells qualitatively and quantitatively by targeting lymphocytes based on the constitution dependent manner. SDT and BYT have been employed as tonic agent and the implication has little been made on the characteristics of the levels of leukocyte subset, such as granulocytes and lymphocytes. In this report, we seek to focus on the identity of each HF formula, comparing to another herbal medicine. The influence of HF on leukocyte and/or lymphocyte subpopulations in human peripheral blood is also discussed. Moreover, some trials have been made for herbal formulae by Lactobacillus and by Yeast Fungus in order to use-up original materials other than traditional hot-water extraction [4].

2. Materials and Methods

2.1. Clinical Findings

2.1.1. Volunteers and Evaluation Methods

Fifteen healthy volunteers aged from 19 to 59 years were recruited and were administered HF for 15 days. Fifteen milliliters of blood were drawn from the forearm vein one hour before the first administration of HF and 15 days after the last HF administration (day 30). All volunteers provided informed consent prior to participation for this trial. This study was approved by Ethics Committee of Kanazawa Medical University.

2.1.2. Herbal Decoction and Assessment for Host Immune Factor

Dried haemopoietic formula SDT and BYT in granules were chosen as representatives of HF [5]-[12]. Dang-Gui-Liu-Huang-Tang (dRHT) and Syao-Chin-Rong-Tang (sCRT) were chosen as controls. A dose of 5.25 g dried powder of each formula (supplied by Tsumura Pharmaceutical Co. Ltd., Japan) was administered two times per day for 15 days. Physiological functions were checked and possible side effects of the drug were inquired for all the subjects to ensure the safety of the trial.

2.1.3. Leukocyte Counts

The assessments including a total number of leukocytes was ordered to count with blood chemical test for the medical diagnosis of public institution (Ishikawa Preventive Medicine Association, Ishikawa, Japan). In the differential counting, 200 cells were counted on a May-Grünewald-Gimsa stained slide, and percentages of lym-

phocytes and granulocytes were determined.

2.1.4. Leukocyte Subset Analyses

The assessments including a total number of leukocytes was ordered to count with blood chemical test for the medical diagnosis of public institution (Ishikawa Preventive Medicine Association, Ishikawa, Japan). In the differential counting, 200 cells were counted on a May-Grunewald-Gimsa stained slide, and percentages of lymphocytes and granulocytes were determined [13] [14].

2.1.5. Lymphocyte Subset Analyses

The whole blood obtained from the subjects was washed twice in phosphate buffered saline (PBS). One hundred micro-liters of the suspensions were stained with 20 µl of fluorescent monoclonal antibodies (anti-human $CD2^+$, $CD4^+$, $CD8^+$, $CD11b^+$, $CD14^+$, $CD16^+$, $CD19^+$ and CD56; antibodies). About 10,000 stained cells were re-suspended in PBS, then surface markers were detected by flow cytometry (FACS Calibur; Becton Dickinson Immnocytometry Systems, CA, USA).

2.1.6. Measurement of Cytokine Expression Levels in Blood Cells

To test whether HF affected the functional maturation of immunocytes within a short period of time, we examined the number of cytokine containing cells using FACS analysis. This method reveals cytokine producing cell by peering off the surface of lymphocyte, enable to assess the cells in a festival evening, compare than serum cytokine level that correspond to paper tips of post festival [15].

Blood cell suspensions were cultured in phorbol 12-myristate 13-acetate (PMA), ionomycin and bovine serum albumin (BSA) (Sigma CO. Ltd., Mo, USA) for 4 - 5 h at 37˚C. Subsequently, the cell suspensions were stained with monoclonal antibodies (Percp-CD3, Percp-CD45, FITC-interferon (IFN)-γ, PE-interleukin (IL)-4, FITC-IL-1β) and analyzed by flow cytometry. All antibodies used in this study were purchased from Becton Dickinson Immunocytometry System (CA, USA).

2.2. Animal Experiment

2.2.1. Experimental Design for Bone Marrow Suppressed Immune-Compromised Mice

In the animal model of immuno-competency reduction, male C57BL/6J mice, aged 8 - 9 weeks, were injected with Mitomycin-C (MMC) (5 mg/kg) to inhibit the bone marrow. Then, HF extracts was administered orally at a dosage of 1 g/kg/day for five consecutive days. tRHT and sCRT were chosen as controls [16]-[24].

1) Recovery of Total Leukocytes and Subsets of Leukocytes

2) Recovery of White Blood Cell by HF

The bone marrow-suppressed mice were administered herbal decoction HF 1g/kg dairy for 5 days and after 1 week later, their blood were withdrawn from their tail vain. Then, the number of leukocytes was counted in Bürker-Türk solution.

3) A Recovery of leukocyte Subsets

Bone marrow-suppressed mice were administered with herbal decoction of dLHT (1g/kg/day) for five days. One week later, the blood from their tail vain was withdrawn. Then the granulocyte and lymphocyte subsets were counted in Bürker-Türk solution.

4) A Recovery of Macrophage Activity, Migration

Cells from peritoneal exudates were collect from the peritoneal cavity of bone marrow-suppressed mice. Phagocytes were purified using adherent technique to get cell suspensions which contained more than 95% of phagocytes. The purified cells were loaded to the upper room of Boyden chamber to test migration ability at a concentration of 1×10^4 cell/ml. Human serum treated at 56˚C for 30 min was for the chemo tactic agent of mouse phagocyte [25].

5) Recovery of Macrophage Activity, Phagocytosis

The same cells suspension was purified by adherent technique for phagocyte, which produces cells contained more than 95% of phagocytes. The purified cells were adjusted to 1×10^4 cell/cm^2 and mixed with latex beads that are 5 µm in granule with fluorescence isochianate. After 90 min of incubation, remained granule were washed out from the glass slide. Number of phagocytic cell and their ability to catch up the latex beads were automatically measured by ACAS system, which outputs the result in a digital form (Adeherent cell activity

evaluating system; Shimazu, Kyoto, Japan).

6) Recovery of Macrophage Activity, Target Cell Killing

The same phagocyte suspension, which contained over 95% of phagocytes, was produced by adherent technique for phagocyte. The purified cells were adjusted to 1×10^5 cell/ml to examine the macrophage activity of killing by the nitroblue tetrazolium (NBT) reduction test [15].

2.2.2. A Recovery of Lymphocyte Activity, Antibody Secreting Cell

The bone marrow suppressed mice were administered herbal decoction of HF (1g/kg/day) for 5 days. One week later, mice were immunized with sheep red blood cells, (2×10^8/mouse) intraperitoneally. Five days later, their spleen cells were collected. Plague-forming cells (PFC) were developed, and the ability of IgM and IgG antibody production was tested by the method reported by Jerne and Nordin [19] [20].

2.2.3. CD Positive Lymphocyte Distribution by HF against Different Constitution

Whole blood obtained from the subjects was washed twice with PBS. One hundred micro-liters of the suspensions were stained with 20 µl of fluorescent monoclonal antibodies (anti-human $CD2^+$, $CD4^+$, $CD8^+$, $CD11b^+$, $CD14^+$, $CD16^+$, $CD19^+$ and $CD56^+$ antibodies). Ten thousands stained cells were re-suspended in PBS to detect surface markers by flow cytometry (FACS Calibur; Becton Dickinson Immnocytometry Systems, CA, USA).

2.2.4. Distribution of Cytokine Producing Lymphocytes in Different Constitution

The blood cell suspensions were cultured with PMA (phorbol 12-myristate 13-acetate), ionomycin and BSA (bovine serum albumin) for 4 - 5 hours at 37˚C. After that, the cell suspensions were stained using the monoclonal antibodies of PE-IL-4, FITC-IFN-γ and FITC-IL-1β. Then they were analyzed by the FACScan (Becton Dickinson Co. Ltd. USA). The antibodies and reagents used in the test were purchased from Becton Dickinson Immunocytometry system (USA).

2.2.5. Estimation of Macrophage Activity by the Examination of Phagocytosis

The peritoneal exudates cells were collect from the peritoneal cavity of bone marrow-suppressed mice. Cell suspensions were purified by adherent technique for phagocyte, getting a suspension which contained over 95% of phagocytes. The purified cells were adjusted to 1×10^4 cell/ml and loaded at the upper chamber of Boyden chamber for test migration. Human serum with treated at 56˚C for 30 min was for the chemotactic agent for mouse phagocyte.

Phagocytic activity and antibody production of macrophages were analyzed using a classical test that could test the total activity of the immune system by examine chemotaxis, phagocytosis and intracellular degradation of macrophage. For identifying antibody-forming cells, plaque-forming cells were detected using heterogeneous erythrocyte; sheep erythrocyte was a target antigen. Peritoneal macrophages were collected and purified in fetal calf serum (FCS)-coated petri-dishes. The cell population was approximately 97% uniform in function and morphology. These cells were applied to the nuclepore-membrane (pore size: 5 µm; Neuro Probe Co. Ltd., Cabin John MD, USA) with a chemotaxis chamber (Neuro Probe Co. Ltd.). After 90 minutes' incubation, the membrane was vigorously washed with saline (37˚C), fixed, and then stained with methylene blue dye. After counting under a microscope for the total field of the membrane, the average number of migrating cells was expressed as cell counts/mm^2.

2.2.6. Phagocytic Ability of Phagocyte

The same cells suspension was purified by adherent technique for phagocyte, which contained over 95% of phagocytes. The purified cells were adjusted to 1×10^4 cell/cm^2 and mixed with latex beads that were 5 um in granule with fluorescence isochianate. After 90 min of incubation, the remained granule were washed out from the glass slide and counting automatically by ACAS system, which outputs digital presentation, for evaluating phagocytes in number and in their ability to catch up the latex beads (Adeherent cell activity evaluating system, Shimazu, Kyoto, Japan). Latex beads in 5 µm with fluorescence were used to test phagocytic activity and *Candida albicans* was cell killing activity. A macrophage-target cell ratio of 1:10 was considered to be optimum. Ten minutes after incubating phagocytes and target cells, intracellular *Candida* cells were cultured on an agar dish with conventional medium 1640 until the next day to perform the colony forming assay. In this way, the phagocytic ability of the macrophages was monitored. To document intracellular killing activity, the same pro-

cedures were performed excepting that the incubation time was changed to 90 minutes.

2.2.7. Antibody Forming Cell Study

Sheep erythrocyte (SRBC), a T-dependent antigen, was used for antibody formation cell study. Ten days after tumor transplantation, each antigen was intra-peritoneally injected. After four and six days, the antibody-forming cells were detected using localized hemolysis in an agar gel. Plaque-forming cells were developed by the method of Jerne and Nordin [19] [20].

2.2.8. Anti-Oxidative Evaluation

1) Animals

Eight week-old female C57BL/6 were purchased from Sankyo Laboratory Service Corporation (Shizuoka, Japan). All mice were kept under specific pathogen-free conditions. Mice food and distilled water were freely accessible for each mouse. Housing temperature and humidity were controlled 25˚C ± 1˚C and 60% (**Figure 1**).

2) Reagents

As for the basic medium, HEPES buffer (HEPES 17mM, NaCl 120mM, Glucose 5mM, KCl 5mM, $CaCl_2$ 1mM, $MgCl_2$ 1mM) was prepared and sterilized by filtration. Phorbol 12-myristate 13-acetate (PMA, Sigma, USA) was diluted to 10^{-6} M by dimethyl sulfoxide (DMSO, Sigma, USA) and used as a stimulant for super oxide anion generation for murine peritoneal exudates cells. Cytochrome-c (Sigma, USA) was diluted to 1mM by HEPES buffer. Since cytochrome-c reduced by super oxide showed maximum absorbance at 550 nm, we used cytochrome-c to measure the amount of super oxide anion generation through spectro-photometrical technique. Oyster Glycogen (type, Sigma, USA) was diluted in the purified water (10% w/v, Wako, Japan) and autoclaved at 120˚C for 20 min. This solution was used for intraperitoneal injection to mice in order to induce peripheral neutrophils into the abdominal cavity.

3) Modified TCM and Other Materials

Agaricus was purchased from Ohbiken Co. Ltd. (Yamanashi, Japan), *Chlorella* was purchased from Taiwan *Chlorella* Co. Ltd. (Taipei, Taiwan) and Propolis was purchased from Epimedix Co. Ltd. (Kanazawa, Japan). The herbal medicine (TCM), Shi-Quan-Da-Bu-Tang and Bu-Zong-Ye-Qi-Tang were purchased from Tsumura Co. Ltd. (Tokyo, Japan) and fermented at Ohbiken Co. Ltd. (Yamanashi, Japan). Propolis was also degradated at

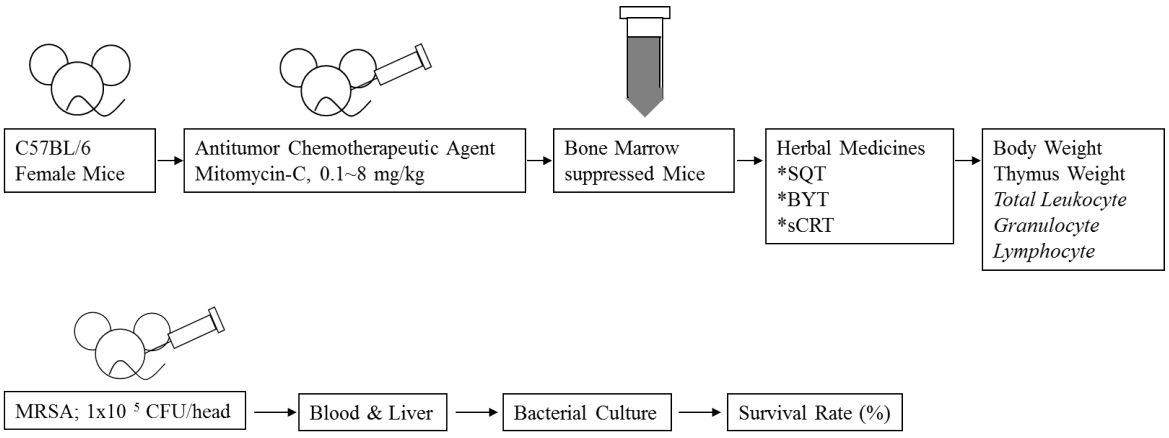

Figure 1. a) The effect on macrophage phagocytosis against latex beads by dHLT after administrating MMC. The procedure of treatment was the same in the text. The macrophage migration capacity was detected by computer analysis, ACAS. Data are expressed as the means ± SE. $^{*}P < 0.05$, MMC versus MMC + dRHT group; b) The effect on macrophage phagocytosis against latex beads by dHLT after administrating MMC. The procedure of treatment was the same in the text. The macrophage migration capacity was detected by computer analysis, ACAS. The data was shown from normal mice; c) The effect on macrophage phagocytosis against latex beads by dHLT after administrating MMC. The procedure of treatment was the same in the text. The macrophage migration capacity was detected by computer analysis, ACAS. The data was shown from MMC-treated mice. Data are expressed as the means ± SE. $^{*}P < 0.001$, MMC versus MMC + dRHT group; d) The effect on macrophage phagocytosis against latex beads by dHLT after administrating MMC. The procedure of treatment was the same in the text. The macrophage migration capacity was detected by computer analysis, ACAS. The data was shown from MMC-treated and dLHLT rescued mice.

Futaba Co. Ltd. (Shizuoka, Japan). Both micronized and not micronized Propolis, *i.e.* native Propolis were insoluble in water, so we used 10% DMSO as a solvent.

4) The Measuring the Amount of Super Oxide Anion Generated by Murine Peritoneal Exudates Cells

Each drug was orally administered to mice (500 mg/kg) for one week. Two milliliters of 10% Oyster glycogen was injected intraperitoneally 10 hours before the assay. Sufficient murine peritoneal exudative cells were induced ten hours after the stimulation. Mice were euthanized by cervical dislocation, murine peritoneal exudates cells (PEC) suspension was centrifuged twice for 5 minutes at 1500 rpm at 4˚C. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred microliters of cytochrome-c and 10 µl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C. The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. An OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ (mmol/ml). In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), an enzyme for its anti-oxidative effect, into our experimental system. The result was as expected that the reduction of cytochrome-c was inhibited after the addition of SOD (**Figure 2**). This showed us that our experimental system could be used properly for measuring the amount of generated super oxide anion.

2.2.9. Statistical Analysis

Data are expressed as means ± standard deviations. The differences between HF-treated and non-treated conditions were compared using a one-tailed analysis of variance. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Clinical Findings

3.1.1 Changes in Cell Number of Total Leukocyte and Subsets

Leukocyte numbers have been counted one hour before and 15 days after the treatment of hemopoitic formula. The cell number measured one hour before the administration was set as 100%. Relative percentage of cell number on the 15th day was calculated. No significant changes were observed in G-group after the administration of BYT. However, significant change was found in L-type group (**Table 1**).

3.1.2. Dividing Subjects into Two Groups, G-Type and L-Type by Granulocyte and Lymphocyte Proportion

The volunteers were healthy subject, with no drastic change for the total number of leukocytes. However, we

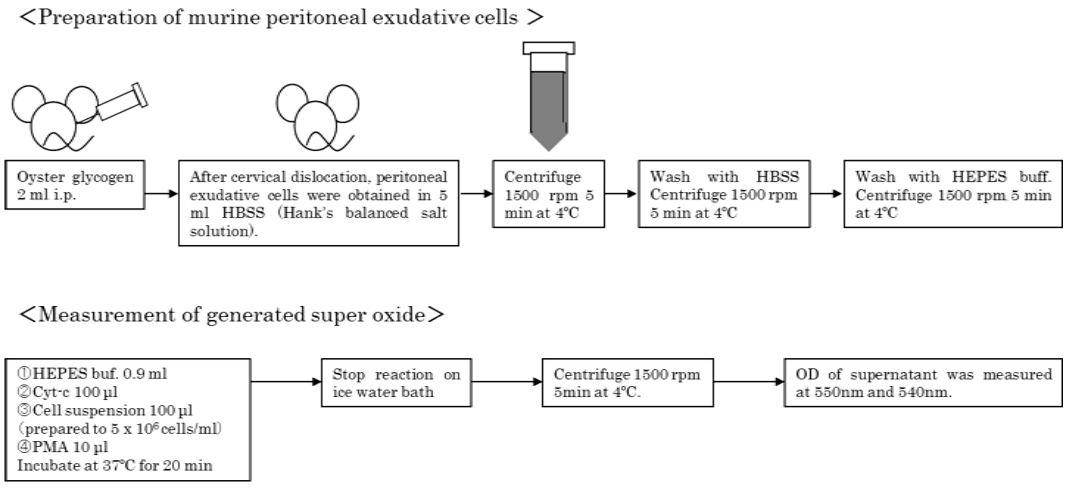

Figure 2. The effect on macrophage migration by dHLT after administrating MMC. The macrophage migration capacity was detected by millipore membrane method separated by 0.25 µm in diameter. Data are expressed as the means ± SE. $^*P < 0.05$, MMC versus MMC + dRHT group.

tried to check the regulative effect of herbal formulae for two different constitution, G-rich type and L-rich type. Analysis that mixed both groups together showed no significant differences in total leukocyte number except that for HF; in G-type group, total number of leukocytes was down regulated by SQT. This was a results of the down regulation of major group of leukocyte, granulocyte.

As for the L-type, no significant changes were found after the treatment of both HFs. In the L-type group, SQT, on the other hand, increased the tonal leukocyte and granulocyte in number, on the contrary to the down regulation for lymphocytes. To further clarify the influence of hemopoietic formula, we divided the subjects into two groups: the G-type group, who had a granulocyte count over 60%, and the L-type group, who had a lymphocyte count over 40%. In the L-type group, lymphocyte counts tended to decrease on day 15, accompanied by an increase in granulocyte numbers by SQT but not by BYT. On the contrary, the granulocyte counts of G-type group tended to decrease on day 15. The decrease of granulocyte count was raised by BYT, but not by SQT on day 15 (**Tables 1-3**).

3.1.3. Lymphocyte Subsets Showed Significant Variation

After HF treatment, cell counts of $CD2^+$, $CD4^+$, $CD8^+$, $CD11b^+$, $CD16^+$, $CD19^+$ and $CD56^+$ were tested to evaluate variations in T cells, B cells, macrophages and NK cells. These values were measured one hour before hemopoietic formula and 15 days thereafter. Our results showed that CD2 and CD4 cells were increased by both BYT and SQT. $CD11b^+$ and $CD14^+$ cell counts, which are closely associated with macrophage activity, increased by SDT in the L-type subjects. In particular, there was a remarkable increase in $CD11b^+$ cell number on day 15. T cell subsets that are closely associated with activity of immature T cells, ($CD2^+$, $CD4^+$ and $CD8^+$), the $CD2^+$ ($P < 0.05$) showed an increase with the treatment of BYT 15 days after administration. The number of

Table 1. Constitution dependent regulation of leukocyte by TCM.

	G-type individual		L-type individual	
	BYT		SQT	
	Before	After	Before	After
Total WBC ($\times 10^3$ μl)	6.95	5.88	3.82	5.04
Lymphocyte (%)	23.6	26.3	44.3	39.2
Granulocyte (%)	69.5	65.4	50.8	55.7
Neutrophil (%)	66.8	62.0	47.3	50.0
Eosinophil (%)	1.9	2.7	2.6	4.8
Basophil (%)	0.8	0.7	0.9	0.9

Table 2. Constitution dependent regulation of lymphocyte by TCM.

CD	G-type individual		L-type individual	
	BYT		SQT	
	Before (%)	After (%)	Before (%)	After (%)
CD2	63.95	76.91	60.25	77.79
CD4	19.75	28.53	31.05	45.14
CD8	38.44	40.37	26.52	28.03
CD11	73.54	70.36	63.37	70.78
CD14	0.03	0.05	0.08	0.01
CD16	66.93	58.02	54.00	41.23
CD19	8.33	8.00	8.39	7.26
CD56	1.68	1.74	1.96	2.41

Table 3. Constitution dependent regulation of cytokine producing cell by TCM.

Cytokine	G-type individual		L-type individual	
	BYT		SQT	
	Before (%)	After (%)	Before (%)	After (%)
IFN-γ	6.95	5.88	3.82	5.04
IL-4	1.9	2.7	2.6	4.8
IL-1β	0.8	0.7	0.9	0.9

$CD19^+$ cells, which is closely associated with B cell activity, was not changed by both HF throughout the trial, neither were the numbers of $CD16^+$ and $CD56^+$ cells (**Table 2**).

3.1.4. Cytokine Producing Cells

To test whether herbal decoction affected the functional maturation of immunocytes in a short time, we investigated the number of cytokine producing/containing cells by FACS analysis. This method reveals cytokine producing cell number by peering off the surface of lymphocyte, enable to express the number of cells in festival evening, compare than serum cytokine level that correspond to the paper tips of post festival. To determine whether HF influences functional maturation of immuno-competent cells, levels of IL-1β-, IL-4- and IFN-γ-expressed T cells were further examined using fluorescence-activated cell sorter analyses. There was a significant increase in the levels of IFN-γ and IL-4 containing cells after administration of SQT. The result revealed that IFN-γ expression, which increased highly on the 15th day after treatment, was different from the expression of IL-1β and IL-4, those on the other hand, exponentially increased on day 15 after the administration of SQT. The augmentation of cytokine expression was confirmed by a classical method in the lymphoid organ, *i.e.* antibody-forming cells and plaque-forming cells. Both HFs down-regulated IL-1β producing cells in both G-type and L-type groups (**Table 3**).

3.2. Animal Test

3.2.1. Recovery of Whole Body Weight by HE

The body weight and thymus weight reduced in bone marrow-suppressed mice, resulting in the reduction of peripheral blood leukocyte to around 40%. After administered each herbal decoction 1 g/kg dairy for 5 days and after 1 week later, their blood were recovered to around 90% of normal value (**Figure 3**).

3.2.2. Recovery of Thymus Weight

The bone marrow-suppressed mice were administered HF 1 g/kg dairy for 5 days, and one week later, their blood was withdrawn from their tail vein. The cell count of the peripheral blood is showed in **Figure 3** shows that the thymus weight decreased to half of normal control after 5 mg/kg of MMC was injected. However, all the three HFs recovered thymus weight to about 70% of the control.

3.2.3. Recovery of CD^+ Cells and Cytokine Producing Cells

CD3, CD4 and CD19 cells of MMC treated mice were recovered to almost normal values after the administration of HFs. As for the functional recovery, IFN-γ and IL-4 producing cells were also recovered by the all three decoction, including HFs and a functionally depressive agent of TCM. In cytokine producing cells, IFN-*r* and IL-4 producing cell were recovered with HF. In all the three HFs tested, cytokine producing cells were recovered with HF and even by the formulae of sCRT (**Figure 4**).

3.2.4. Recovery of Macrophage Activity, Phagocytosis by HF

Figure 5 shows that MMC clearly suppressed the phagocytic activity of mice both in number and function. After the treatment of HF, the mice recovered their phagocytic activity to normal range. With a precise observation, the recovery activity was different between SQT and BYT. SQT was the strongest HF among the four formulae to augment in number and function of phagocytes. On the other hand, the augmentation by BYT was less than that by SQT (**Figures 5-9**).

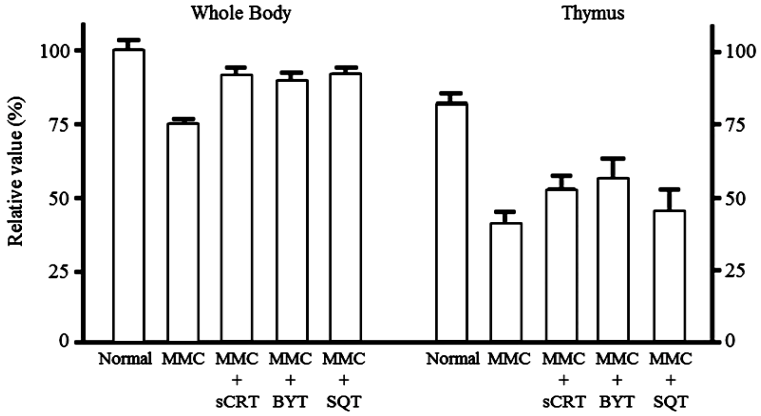

Figure 3. The effect on macrophage killing activity against revealed with NBT reduction by dHLT after administrating MMC. The procedure of treatment was the same in the text. The killing activity was detected by Nitro-blue Tetrazolium. Data are expressed as the means ± SE. $^{*}P < 0.001$, MMC versus MMC + dRHT group.

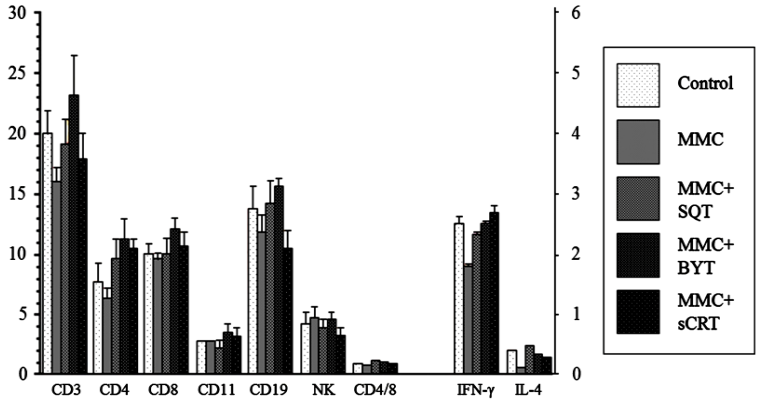

Figure 4. The experimental infection test were performed by the mice devided by 4 group. The groups were consisted by normal, MMC-treated, MMC-treated + dHLT, MMC-treated + dRHT plus chemotherapeutic agency vancomycin.

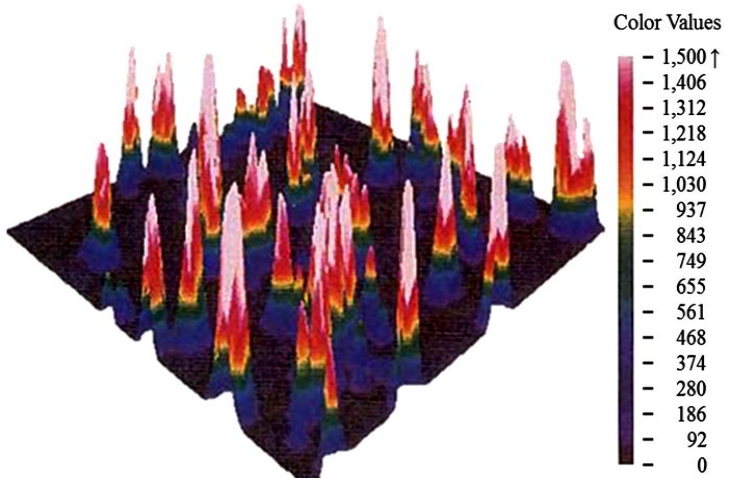

Figure 5. The number of MRSA counting in the organ that experimentally infected by 5×10^6 of microorganisms. Data are expressed as the means ± SE. $^{*}P < 0.001$, MMC versus MMC + dRHT group.

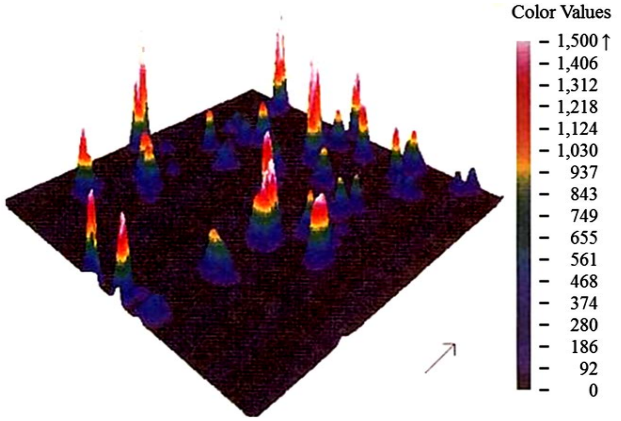

Figure 6. The site analysis that augmented by dHLT after inhibited by MMC. A granulocyte and lymphocyte were separate and adoptively transferred to MMC treated recipient mice. Then 5 × 1 MRSA were challenged and followed up their survival rate.

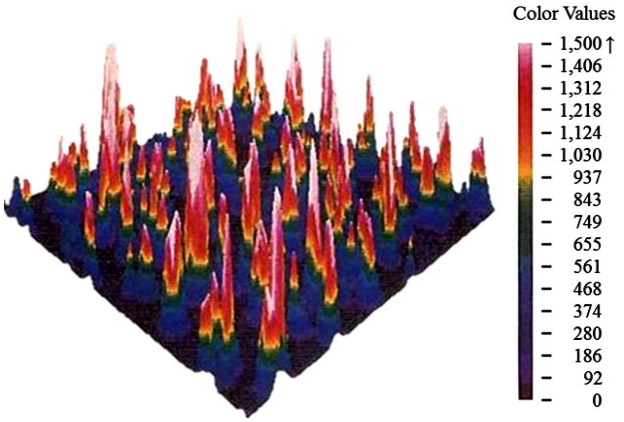

Figure 7. Human bed side test was done for stroke and/or heart infarction patient by dRHT. The herbal decoction, 8 g/head of dRHT was administered daily for at least one month. Then MRSA were chased by collecting sputa from the patient. The blood chemical data were shown in the figure.

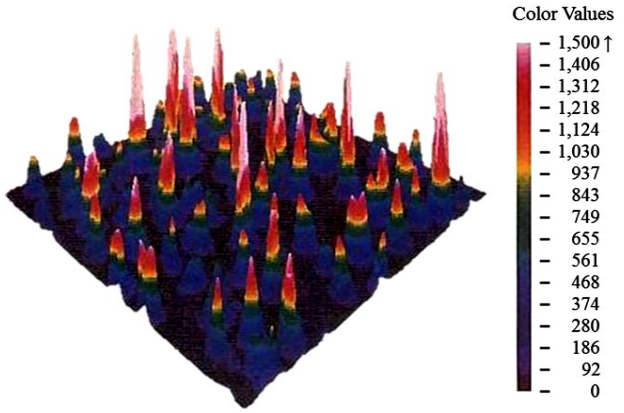

Figure 8. Human bed side test was done for the patient who were suffering heat infarction positive with administrating dRHT. The herbal decoction, 8 g/head of dRHT was administered daily for at least one month. Then MRSA were chased by collecting sputa from the patient. The MRSA containing from the patient sample were shown in the figure.

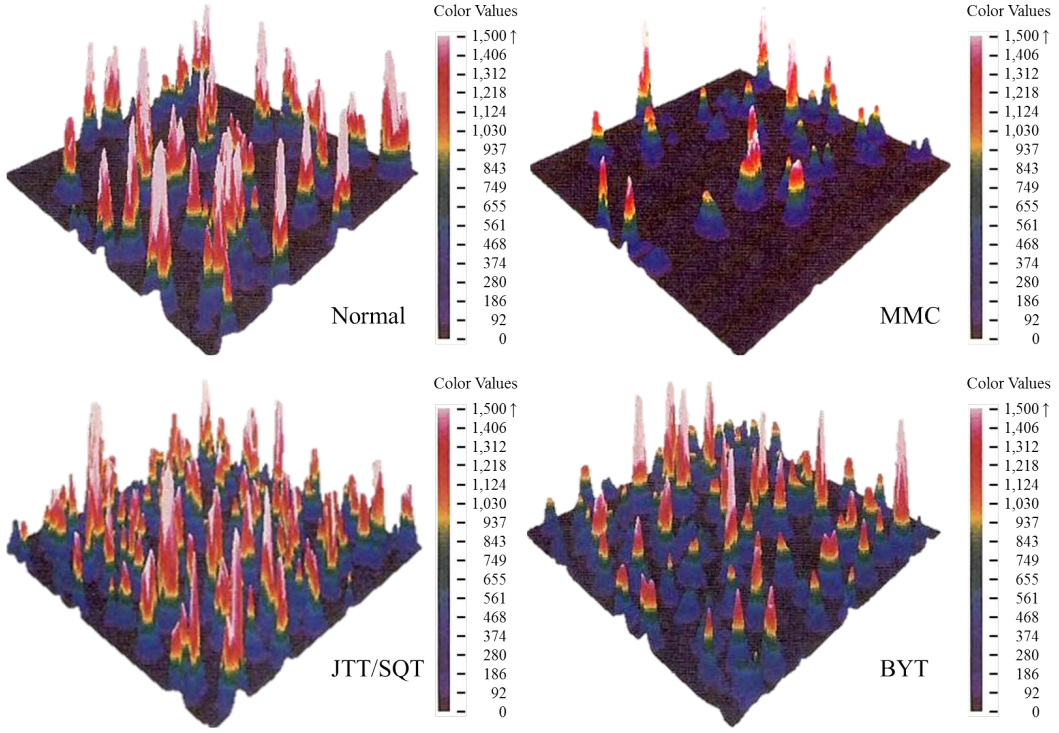

Figure 9. The clinical strain of multi-drug resistant bacteria were collected and incubated by various dose of sterile dRHT. After incubationg overnight, each bacteria was tested their drug sensitivity. The strains of clinical origin were *H. influluenzae* I-105, *H. influluenzae* I-147, *E. coli* ML 4901/Rms212, *E. coli* ML 4901/Rms213, *E. coli* ML 4901/Rte16, *E. coli* ML. 4901/Rms149, and *P. aeruginosa* PAO 0214/pMG26.

3.2.5. Recovery of Lymphocyte Activity, Antibody Secreting Cell

The bone marrow-suppressed mice were administered herbal decoction HF for five days. One week later, mice were immunized with sheep red blood cells, (2×10^8/mouse) intraperitoneally. Four and six days later, their plague-forming cells (PFC) were developed. The ability of IgM and IgG antibody production was tested by the method reported by Jerne and Nordin [19] [20]. In this mouse model, MMC did not reduce the antibody forming cells significantly but the tendency was the same as shown in the former section. In this test, B was the most effective than that of A. BYT was the strongest material to augment antibody secreting cell among the four formulae (**Figure 10**).

3.2.6. Phagocytic Activity of Macrophage

So as to detect the supportive effect and important immunological stimulation by tonic agent, Bu-Ji, we traced the augmentation pattern of each remedy. As results of this trial, the phagocytic patterns by tonic agents, Bu-Ji, were clearly different from MMC-treated mice. Moreover, augmentation of phagocytes were different between each HF. SDT was prominent in activating phagocytes quantitatively and qualitatively compared to BYT.

We showed the diversity in the recovery pattern of HFs. Famous tonic remedies in China and Japan, SDT, strongly recovered phagocytic activity in compromised hosts, but the recovery by BYT was much less than that by SDT. The recovery level of phagocytic activity by sCRT was between SDT and BYT (**Figures 5-9**, **Table 4**).

3.2.7. The Amount of Generated Super Oxide Anion

The amount of generated super oxide anion was calculated in the formula shown above. The generated super oxide anion after one week administration of *Agaricus* and *Chlorella* were 2.64 and 1.95×10^{-5} mmol/ml, respectively, whereas that was 2.85×10^{-5} mmol/ml in control group. The generated super oxide anion after one week administration of herbal medicine, SDT, BYT and sCRT were 1.24, 1.25 and 2.88×10^{-5} mmol/ml, respectively. The generated super oxide anion after one week administration of Propolis was 2.55×10^{-5} mmol/ml. All these drugs, except for sCRT, decreased super oxide anion generation after administration for one week in

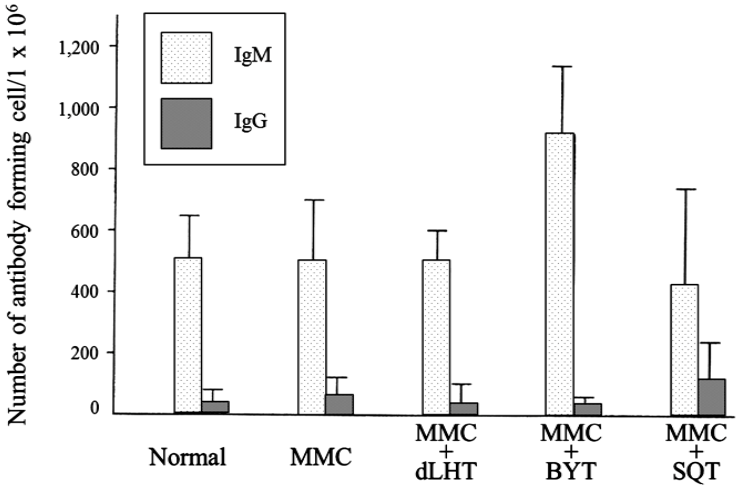

Figure 10. Recovery of antibody secreting cell.

Table 4. Relative activities of macrophage phagocytosis.

	Phagocytosi	
	Positive Cells/10^6 cells	
	Law active (%)	High active (%)
Normal	52 (100)	45 (100)
MMC	34 (65)	5 (11)
MMC + SQT	108 (207)	59 (131)
MMC + BYT	93 (178)	12 (26)
MMC + GNT	81 (155)	46 (102)
MMC + dLHT	73 (140)	43 (95)

mice (**Figure 11**).

3.2.8. The Comparison of Generated Super Oxide Anion between the Fermented and Not Fermented Herbal Medicine

Since the antioxidative effects of herbal medicine were demonstrated, we investigated the way to reinforce this effect. The fermentation is one of the possibilities. Since the fermentation is preceded by bacterial digestion and degradation, less efficient constituents would be lost than commonly used extraction by hot water. Therefore, we decided to ferment the herbal medicine by yeast (*Saccharomyces cerevisiae*), expecting the enhancement of its antioxidative effects. The generated super oxide anion after one week administration of fermented herbal medicine TCM, SDT, BYT and sCRT were 0.62, 0.84 and 1.50×10^{-5} mmol/ml, respectively. All the fermented herbal medicine decreased super oxide anion generation in compare with their corresponding unfermented ones (**Figure 12**, **Table 5**).

3.2.9. The Comparison of Generated Super Oxide Anion between the Pharmented and Original Propolis

The antioxidative activity of Propoils has been demonstrated, however, the particle of native Propolis was seen to be gross. In order to reinforce its antioxidative activity from physical constructive view point, we tried to micrified Propolis into 0.5 μm, expecting enlarged attachment area with reaction mixture. The generated super oxide anion after one week administration of micrified Propolis was 2.52×10^{-5} mmol/ml, whereas that of non-micrified Propolis was 2.55×10^{-5}mmol/ml. The antioxidative activity was slightly enhanced by micrifying the drug (**Figure 11**, **Table 6**).

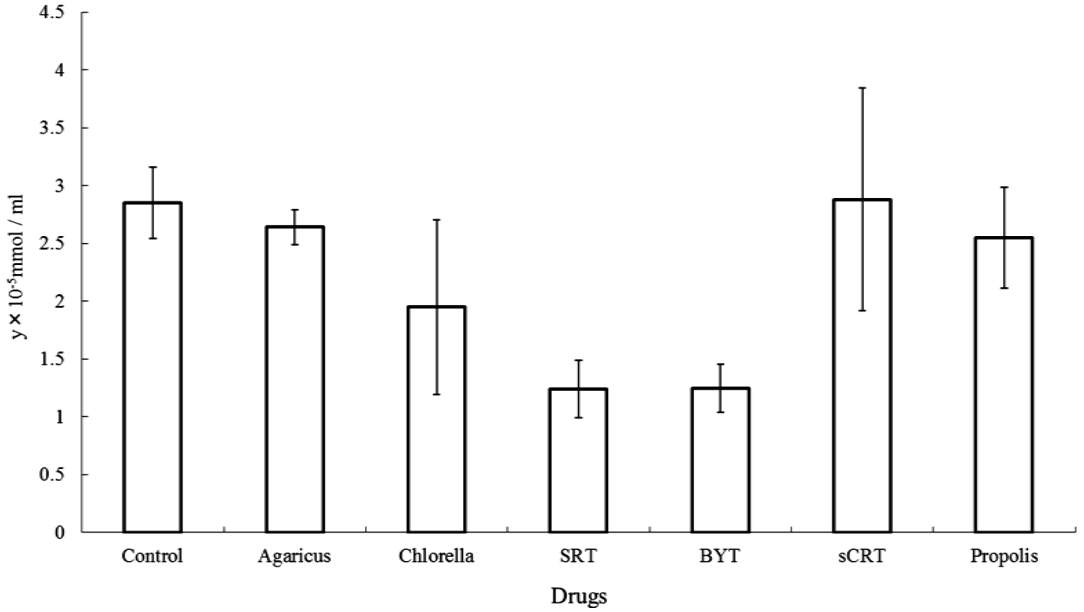

Figure 11. Anti-oxidative activity accessed by peritoneal macrophage.

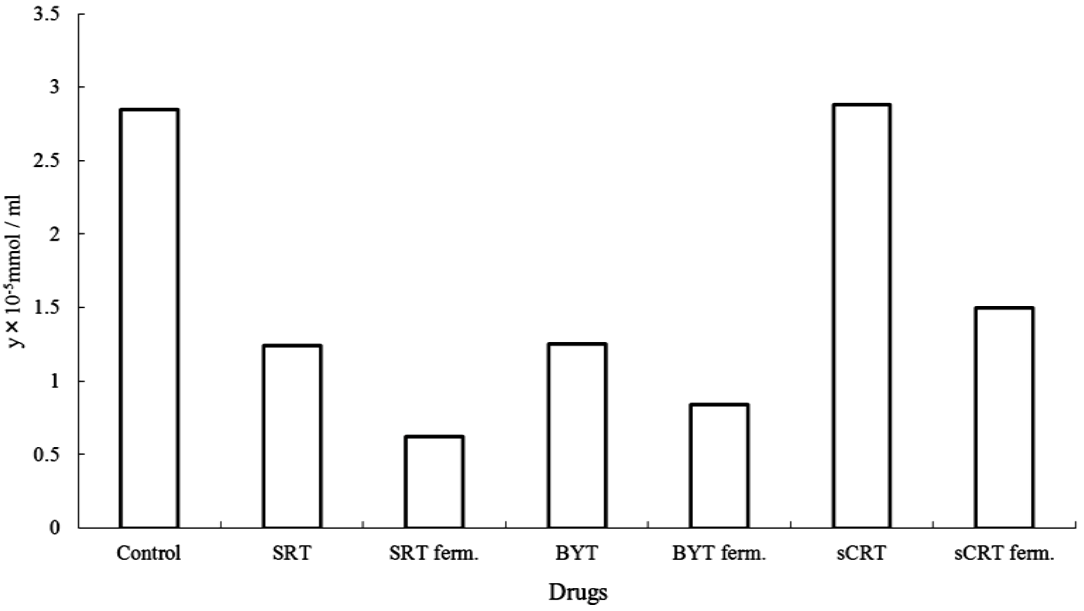

Figure 12. Effect of anti-oxidative activity on fermentation by yeast.

Table 5. Anti-oxidative activity by macrophages.

Materials	Generated O_2^- ($\times 10^5$ mmol/ml)
Agaricus burazei	2.64
Chlorella pirenoidosa	1.95
SQT	1.24
BYT	1.25
sCRT	2.88
Propolis	2.55
Control	2.85

Table 6. Plaque forming/antibody secreting cell.

Group Number	PFC/10^6 Spleen Cells
1 Normal Mice	543 ± 36
2 MMC Control	203 ± 84
3 MMC + SQT	486 ± 83*
MMC + fSQT	656 ± 72**
4 MMC + BYT	553 ± 62*
MMC + fBYT	776 ± 84**
5 MMC + dLHT	486 ± 55*
6 MMC + sCRT	352 ± 46*

*$p < 0.01$ comparing to MMC control. **$p < 0.05$ comparing to non-fermented formulae.

3.2.10. Antibody Forming Cell Study by Parented TCM

Sheep erythrocyte (SRBC), a T-dependent antigen, was used for antibody formation cell study. Ten days after tumor transplantation, each antigen was intra-peritoneally injected. After four and six days, the antibody-forming cells were detected using localized hemolysis in an agar gel. Plaque-forming cells were developed by the method of Jerne and Nordin [19] [20].

The fermentation is preceded by bacterial digestion and degradation, less of the efficient constituents would be lost than commonly used extraction by hot water. Therefore we decided to ferment the herbal medicine by yeast (*Saccharomyces cerevisiae*), expecting the enhancement of lymphocyte activating effects through antibody forming cells. The antibody forming cells after one week's administration of fermented SQT and BYT were 135% and 140%, respectively. All the fermented herbal medicines from HF increased PFC (**Table 6**).

4. Discussion

Our investigation clarified how hemopoitic formula, also known as tonic agent and Bu-Ji, influenced the immune system (e.g. leukocyte, granulocyte and lymphocyte subsets in particular).

We quantified CD positive cell counts as indicators of T cells, B cells, macrophages and NK cells. For qualitative and quantitative evaluation, we examined the cytokine expression levels, and directly measured the expression levels of cytokine-containing cells in peripheral blood, eliminating possible artificial factors that could arise from culturing in test tubes or changes in net value by catalyzation. To avoid any possible influence from the circadian rhythm, we obtained the whole blood from all donors at the same time.

In this investigation, we confirmed that HF quantitatively and qualitatively regulated leukocytes, granulocytes, lymphocytes and their subsets. The increase of $CD2^+$, $CD4^+$, $CD8^+$, $CD11b^+$, $CD16^+$, $CD19^+$ and $CD56^+$ cell counts as well as the levels of IL-1β, IL-4 and IFN-γ in blood cells suggested that hemopoitic formula might enhance the activities of humoral and cellular immunities, as well as NK cells. We also observed that levels of cytokine producing cells, in particular, increased rather than CD-positive lymphocytes, showing that HF augmented lymphocyte production qualitatively than quantitatively. Moreover, HF activated both CD11b cells and IL-1β producing cells, suggesting the activation of phagocyte cells both in number and in function. Consequently, these data further demonstrated that HF acted in macrophages in the same manner as *Mycobacterium tuberculosis* that had cell walls constructed of waxy substances [13].

In previous reported about hot-spring hydrotherapy and acupuncture, we had proposed that immune system regulation was an important factor for evaluating CAM. Since other substances, such as endotoxin and waxy substances from *Mycobacterium tuberculosis*, similar to Proplis, were known for augmenting host immune responses. This time, we decided to focus solely on Propolis. A possible explanation for immune enhancement could be the activation of the circular system and/or autonomic nervous system, although the details of the mechanism remained unclear. Further research regarding to the mechanism was necessary.

Abo *et al.* reported that granulocyte count was increased by the excitation of the sympathetic nervous system, while lymphocyte count was increased by excitation of parasympathetic nervous system [14]-[18]. Our data also showed that granulocyte count was decreased in subjects with a high granulocyte count, while lymphocyte count

was increased in the same subjects. The lymphocyte count, however, was decreased in subjects with a high lymphocyte level, while granulocyte count was increase in the same subjects. In other words, the subjects dominated by the sympathetic nerve could release stress, whereas the sympathetic activity of subjects who were dominated by the parasympathetic nerve might be excited by hemopoietic formula. This way, the cell counts appeared to converge at appropriate levels after hemopoietic formula. Finally, in order to determine whether the elevation of leukocyte counts resulted from an infection triggered by hemopoitic formula or not, the subjects were followed up for 8 days after the last administration of hemopoitic formula. During that period, we could not observe any infectious signs such as pyodermitis, fever, or enhancement of C-Reactive Protein (CRP). The value of CRP was 0.57 g/dl to 1.23 g/dl in our subjects, suggesting very mild inflammatory responses, which showed that hemopoitic formula did not cause infection. Since the meridian may influence cells throughout the body and may pass through every organ system, hemopoitic formula stimulation might provide maximum benefits without harmful side effects [21]-[25]. As an immune-enhancer, hemopoitic formula merits further investigation as a possible treatment for acquired immunodeficiency syndrome, chronic fatigue syndrome and other disorders that have been concerned throughout the world [26]-[28].

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Abbreviations

BYT; Bu-Zong-Ye-Qi-Tang: a famous TCM formula for augment blood cell in number and function.
CAM: Complementary and alternative medicine, beside the western medicine, there are many traditional medicine and/or health promoting menu all over the world.
CD; Cluster of differentiation. Each lymphocyte has name that expressed CD number, for example CD2, CD4, etc.
FCM; Flow Cytometry
G-rich type; The individual that exhibit over 60% of granulocyte in peripheral blood, finding many in young gentleman.
L-rich type; The individual that exhibit over 40% of lymphocyte in peripheral blood, finding lot in ladies and senile
SDT; Shi-Quan-Da-Bu-Tang: a famous TCM formula for augment blood cell in number and function.
sCRT; Shao-Chin-Rong-Tang: a famous TCM formula for allergic syndrome, suppressing lymphocyte in number and function