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Fermented Black Turmeric Designed by Lactobacillus Rearranged Leukocyte Subsets and Anti-Oxidative Activity

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Abstract

A plant fermentation was carried out by Yeast and *Lactobaccilli* against fermented black turmeric, *Kaempferia parviflora* (FBT). These materials were proved by as safe in animal safety experiment. We tried to investigate changes of immune-competent cells that commonly utilized FBT, including after administration of immno-suppressed animals, the effects by FBT on the regulated effect on the cells were evaluated. Our results showed that FBT augmented the level of lymphocytes in number, while FBT regulated the level of granulocytes in both number and function. In our clinical study with 20 healthy volunteers, granulocyte and lymphocyte ratio suggesting their constitution as neutral in peripheral blood were increased significantly 30 days after the administration of FBT in rodents, and compromised host was prepared with cancer chemotherapeutic agent (Mytomycin-C). Our observations showed against intracellular parasite, and that FBT augmented intercellular pathogen through humoral immunity. We discussed the significance and mechanism of FBT on the level of leukocyte subsets in number and function that were considered to be potential indicators for the activation of the compromised host. We also proposed an idea that FBT exhibited tonic effects via activating complement components. The evidences were shown by immune-electrophoretic method. Moreover, we tried to access further to the anti-oxidative activities of this FBT. This modification brought to the significant lift up for antibody producing cells and anti-oxidative activity for phagocytic cells.

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Keywords

Family Turmeric Lactobacillus, Fermentation, *Lactobacilli,* Cancer, Chemotherapeutic Agent, Compromised Host, Complement, Alternative Complement Activation

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, preparing special molecule for both digestive and easy for activate human complement component.

The activated complement components bound to regulate function of all the leukocytein humanimmune system [1]-[7]. Dietary and fermented formula holds a 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 [8]. In this study, we hypothesize that FBT may influence immuno-competent cells qualitatively and quantitatively FBT targeting lymphocytes based on the constitution dependent manner. FBT has 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 FBT formula, comparing to another herbal medicine. The influence of FBT on leukocyte and/or lymphocyte subpopulations in human peripheral blood was also discussed. Moreover, Fragmentation of black turmeric has been made by Lactobacillus and by Yeast Fungus in order to induce active component from original material other than dairy use [9] **Figure 1**, **Figure 2**.

2. Materials and Methods

2.1. Single Shot and Multiple Shot Toxicity

Ten female seven-week-old ddY mice, were used for the acute oral toxicity study. The tests were carried out according to Ethics of the Organization for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were housed at 24˚C ± 1˚C, 50% relative humidity. Both conventional and charged water were suspended in sterile and administered to mice in free supplemental system, calculating daily consumption. Mice

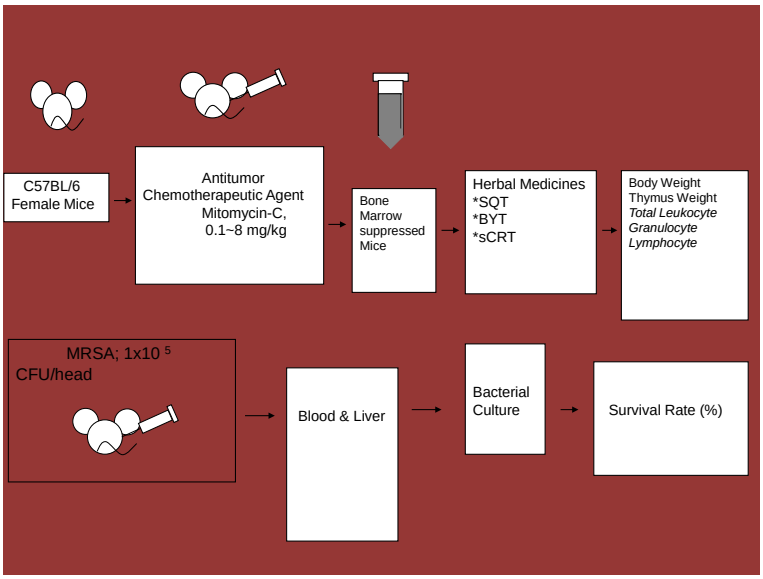

Figure 1. Experimental model for the study FBT in immuno-compromised mice.

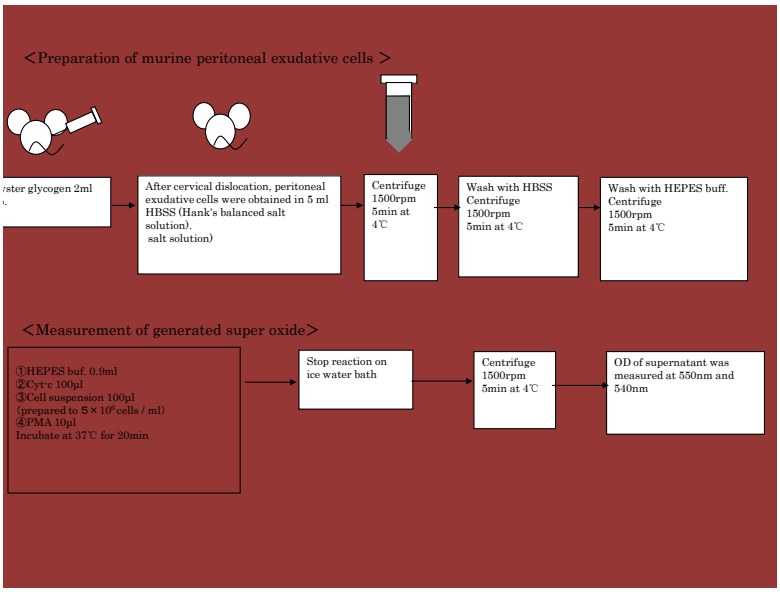

Figure 2. The experimental procedure for accessing anti-oxidative of the mice macrophage.

were weighted at 0 - 7 days after administration, and clinical observations were made once a day. Necropsy was performed on all mice seven days after administration.

2.2. Experimental Model 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) (5mg/kg) to inhibit the bone marrow. Then, FBT extracts was administered orally at a dosage of 1 g/kg/day for five consecutive days. *Conventional black turmeric* and *fermented black turmeric* were chosen as controls (**Figure 1**).

2.2.1. Recovery of Total Leukocytes and Its Subsets

The bone marrow-suppressed mice were administered herbal decoction FBT 1 g/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.

2.2.2. Regulation of Leukocyte Subsets

Bone marrow-suppressed mice were administered with herbal decoction of FBT (1 g/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.

2.2.3. 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 [10]-[18].

2.2.4. Augmentation 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).

2.2.5. A Recovery of Lymphocyte Activity, Antibody Secreting Cell

The bone marrow suppressed mice were administered herbal decoction of FBT (1 g/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].

2.2.6. CD Positive Lymphocyte Distribution by FBT 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).

2.2.7. 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).

2.2.8. Estimation of Macrophage Activity FBT 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.

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 FBT 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 (Adherent cell analyzing 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 procedures were performed excepting that the incubation time was changed to 90 minutes.

2.2.9. 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-form-

ing cells were detected using localized hemolysis in an agar gel. Plaque-forming cells were developed FBT the method of Jerne and Nordin [19] [20].

2.3. Anti-Oxidative Evaluation

2.3.1. Experimental 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%.

2.3.2. Reagents and Preparation

As for the basic medium, HEPES buffer (HEPES 17 mM, NaCl 120 mM, Glucose 5 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM) 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 1 mM 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 II, 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 (**Figure 2**).

2.3.3. The Assessing 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. This showed us that our experimental system could be used properly for measuring the amount of generated super oxide anion.

2.4. Statistical Analysis

Data are expressed as means ± standard deviations. The differences between FBT-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. Animal Test for FBT

3.1.1. Single and Multiple Dose Toxicity study for *Conv.* BT and *Fermented* BT

Ten female seven-week-old ddY mice, were used for the acute oral toxicity study. The tests were carried out according to Ethics of the Organization for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were housed at 24˚C ± 1˚C, 50% relative humidity. Both conventional and charged water were suspended in sterile and administered to mice in free supplemental system, calculating daily consumption. Mice were weighted at 0 - 7 days after administration, and clinical observations were made once a day. Necropsy was performed on all mice seven days after administration.

No deaths or abnormalities of body weight, water and food consumption, or coat condition were observed in

the treated mice. Necropsy evaluation of the mice did not reveal any significant differences in thymus, liver, spleen, kidney, adrenal gland and testicle weights between the control group and both conventional water and charged and activated water.

3.1.2. Recovery of Whole Body Weight by FBT

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

3.1.3. Recovery of Thymus Weight by FBT

The bone marrow-suppressed mice were administered FBT 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 4**. **Table 1** shows that the thymus weight decreased to half of normal control after 5 mg/kg of MMC was injected. However,

Table 1. Proteins of the complement system.

Protein	Molecular Weight	Plasma Concentration	
Classical Pathway			
C1q	400,000	65	μg/ml
C1r	190,000	50	μg/ml
C1s	88,000	40	μg/ml
C4	200,000	640	μg/ml
C2	117,000	25	μg/ml
C3	185,000	1400	μg/ml
Alternative Pathway			
Factor B	93,000	200	μg/ml
Factor D	23,000	2	μg/ml
C3	185,000	1400	μg/ml
Membrane Attack Pathway			
C5	200,000	80	μg/ml
C6	128,000	75	μg/ml
C7	121,000	55	μg/ml
C8	154,000	55	μg/ml
C9	79,000	60	μg/ml
Regulatory Proteins			
C1 Inhibitor	85,000	20	μg/ml
C4b Binding protein	570,000	250	μg/ml
Carboxypeptidase N	310,000	50	μg/ml
Factor H	150,000	500	μg/ml
Factor I	80,000	35	μg/ml
Properdin	180,000	25	μg/ml
S-Protein	71,000	600	μg/ml

all the three FBTs recovered thymus weight to about 70% of the control.

3.1.4. Recovery of CD⁺ Cells and Cytokine Producing Cells by FBT

CD3, CD4 and CD19 cells of MMC treated mice were recovered to almost normal values after the administration of FBTs. As for the functional recovery, IFN-γ and IL-4 producing cells were also recovered by FBT. The all three decoction, including FBTs and a functionally depressive agent of TCM. In cytokine producing cells, IFN-γ and IL-4 producing cell were recovered with FBT. In all the three FBTs tested, cytokine producing cells were recovered with BT and even fermented BT.

3.1.5. Recovery of Macrophage Activity, Phagocytosis by FBT

The figure shows that MMC clearly suppressed the phagocytic activity of mice both in number and function. After the treatment of FBT, the mice recovered their phagocytic activity to normal range. With a precise observation, the recovery activity was different between BT and FBT, and were the strongest FBT among the four formulae as to augmentation in number and function of phagocytes. On the other hand, the augmentation by BT was less than that by FRT

3.1.6. Recovery of Lymphocyte Activity, Antibody Secreting Cell by FBT

The bone marrow-suppressed mice were administered herbal decoction FBT 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 FBT, 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. FBT was the strongest material to augment antibody secreting cell among the two formulae.

3.1.7. Phagocytic Activity of Macrophage by FBT

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

We showed the diversity in the recovery pattern of FBTs. Famous tonic remedies in China and Japan, FBT, strongly recovered phagocytic activity in compromised hosts, but the recovery by FBT was much less than that by FBT. The recovery level of phagocytic activity by FBT was between conventional black turmeric and Fermented one.

3.2. The Amount of Generated Super Oxide Anion Regulated by FBT

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 BT and FBT 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 FBT, decreased super oxide anion generation after administration for one week in mice.

3.3. The Comparison of Generated Super Oxide Anion between the Fermented and Non Fermented Black Turmeric

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, FBT, FBT 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 3**).

3.4. The Comparison of Generated Super Oxide Anion between the Fermented and Original Black Turmeric

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.

3.5. Antibody Forming Cell Study by Black Turmeric Derivatives

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 FBT. 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 FBT were 135% and 140%, respectively. All the fermented herbal medicines from FBT increased PFC.

4. Clinical Findings

4.1. Changes in Cell Number of Total Leukocyte and Subsets by FBT

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 FBT. However, significant change was found in L-type group (**Table 2**).

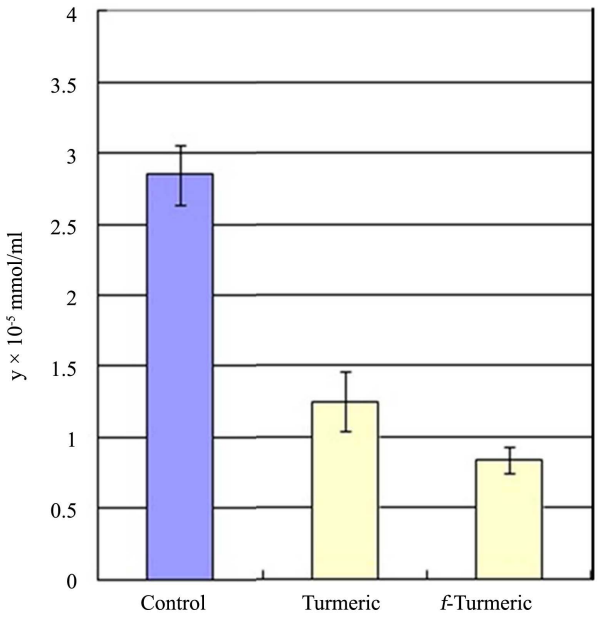

Figure 3. Anti-oxidative activity by fermented black turmeric and conventional one.

Table 2. Constitution dependent regulation of leukocyte by *Black Turmeric*.

	G-type individual		L-type individual	
	Conv. B. Turmeric		*f-B. Turmeric*	
	Before	**After**	**Before**	**After**
Total WBC ($\times 10^3$ µl)	6.44	5.78	3.47	5.65
Lymphocyte (%)	25.6	26.8	43.6	38.7
Granulocyte (%)	67.5	68.5	54.8	57.5
Neutrophil (%)	65.5	64.3	44.6	53.1
Eosinophil (%)	1.5	2.9	2.5	4.2
Basophil (%)	0.5	0.6	0.8	0.8

4.2. 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 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 FBT; in G-type group, total number of leukocytes was down regulated by *conventional black turmeric*. 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 FBTs. In the L-type group, FBT, 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 *conventional black turmeric* but not by FBT. On the contrary, the granulocyte counts of G type group tended to decrease on day 15. The decrease of granulocyte count was raised by *fermented black turmeric*, but not by *conventional black turmeric* on day 15 (**Table 3**).

4.3. The Complement System-Another Stage for Focusing by Fragmented Polysaccharide by FBT

We would like to focus on another important factor of immunological component, complement. These proteins are composed of at least 9 components. These proteins are famous for its defensive activity against infections organisms as in the defense immunity. However, we had found that the complement had worked when we introduced fragmented/fermented polysaccharide as complement activator, so called alternative pathway conjunct to Alternative Medicine. So in this chapter, we would like to show the nature of complement and activated mechanism that lead to the activation of all the physical activities through the augmentation of complement receptor positive structure cells. Activation of the complement system results in a cascade of interactions of these proteins, leading to the generation of products that have important biologic activities and that constitute an important humoral mediator system involved in inflammatory reactions. First, coating of particles, such as bacteria or immune complexes, with certain components of complement facilitates the ingestion of the particle by phagocytic cells (opsonic function of complement). Second, the activation event generates many fission products of complement proteins for which specific receptors exist on a variety of inflammatory cells, such as granulocytes, lymphocytes, and other cells. Binding of these complement-derived products to such receptors results in biologic activities such as chemotaxis and hormone-like activation of cellular functions (inflammatory function of complement) [21] [22] (**Figure 4**).

4.3.1. Pathways of Complement Activation and Complement Proteins by FBT

The complement component can activate by two separate pathways: the classical and the alternative pathways.

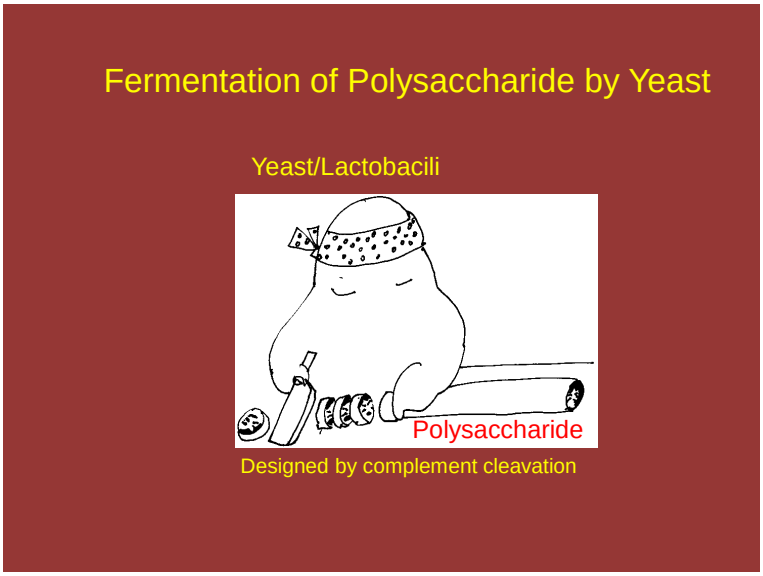

Figure 4. An illustrative imaging for degradation of polysaccharide by microorganism.

Table 2. Constitution dependent regulation of lymphocyte by *Black Turmeric*.

CD	G-type individual		L-type individual	
	Conv. B. Turmeric		*f-B. Turmeric*	
	Before (%)	After (%)	Before (%)	After (%)
CD2	6.66	76.76	60.43	77.65
CD4	19.54	28.44	31.43	45.67
CD8	37.65	42.57	26.38	28.63
CD11	73.77	72.68	63.45	69.54
CD14	0.03	0.06	0.06	0.07
CD16	67.65	58.55	54.24	46.67
CD19	8.45	8.21	8.41	7.95
CD56	1.57	1.88	1.78	2.87

Both pathways lead to a common terminal pathway referred to as the pathway of membrane attack complex. Twenty plasma proteins are now known to be constituents of these pathways. These proteins can be divided into functional proteins, which represent the elements of the various pathways, and regulatory proteins, which exhibit each function. The blood level of the proteins in normal human varies a broad range. They are synthesized in the liver but also by cells of the lympho-reticular system, such as lymphocytes and monocytes. Both the classical and the alternative complement pathways can be organized into various operational units: initiation, amplification, and membrane attack. Following an initial recognition event, which leads to initiation of the pathway, an amplification phase takes place that involves the action of proteases and the recruitment of additional molecules; this is followed by a terminal phase of membrane attack during which the cell dies. The recognition unit for the classical pathway, C1, is composed of three separate proteins, Clq, Clr, and Cls. The initiation of this pathway of complement typically involves the reaction of antibody with antigen, which may be soluble or on the surface of a target cell. This antigen-antibody reaction allows the binding of Clq to two or more Fc regions of certain IgG subclasses (IgG1, IgG2, IgG,) or IgM. Activators of the classical pathway. The ultra structure of Clq has been demonstrated by electron microscopy to consist of six subunits similar to a bouquet of six flowers. The central

stalks of Clq resemble collagen in primary and secondary structure. Upon binding of one Clq molecule to the Fc regions of two or more antigen-bound antibody molecules, Clr proenzymes are activated. The chemical basis of this activation is the cleavage of a peptide bond by an autocatalytic mechanism, leading to the formation of activated Clr, a protease that subsequently cleaves the proenzyme Cls. Thus, the binding of Clq to an immunoglobulin in complex with the antigen represents the recognition event of the classical pathway, resulting in the activation of Clr and Cls. The final result is the generation of an enzymatically active component, Cls, which will cleave and thereby activate the next proteins in the cascade, leading to amplification of the recognition event. The other activation process, polysaccharide molecule also hits the complement component. Therefore, some polysaccharide molecule hit the complement component in the manner of alternative pathway. Thus, U-164 derivatives activated human complement component and shown by immune elecrophoretic methods.

The enzymatic protein Cls has two physiologic substrates, C4 and C2. C4 is cleaved by Cls into C4a, one of the three anaphylatoxins (molecules that promote increased vascular permeability and smooth muscle contraction), and C4b, which binds to the target cell surface. Cls also cleaves C2 when C2 is in complex with C4b. Cleavage of C2 generates C2b, which is released, and C2a, which remains bound to C4b. The bimolecular complex C4b, 2a is a protease that cleaves C3 and therefore is called C3 convertase. Cleavage of C3 by the C3 convertase generates two important biologically active peptides, C3a (another anaphylatoxin) and cab, which attaches to target cell surfaces and can bind to C5. C5, when in complex with C3b, can be cleaved by the C3 convertase (then referred to as C5 convertase). The C5 convertase hydrolyzes C5, which generates the C5a anaphylatoxin and C5b. C5b is the nucleus for the formation of the membrane attack complex. Immediately following their generation, C3b and C4b exhibit a unique transient ability to covalently bind to target cells ("metastable binding site"). This property has reentry been shown to be due to an intramolecular thioester bond that is present between the sulfhydryl group of a cysteine residue and the gamma carbonyl group of a glutamine residue on C3 and C4. Upon activation of C3 or C4, this thioester becomes highly reactive and can react with a cell surface hydroxyl or amino group. This results in the covalent attachment of C3b or C4b to the target cell. An additional function of the thioester bond is its hydrolysis by water, occurring during activation of the alternative pathway as described below.

The alternative pathway can be activated when a molecule of C3b is bound to a target cell. This C3b molecule combines with the plasma protein Factor B, which is a zymogen, and which, when bound to C3b, can be activated by the plasma protein Factor D by cleavage into two fragments, Ba and Bb. The Bb fragment, which contains the active enzymatic site, remains bound to C3b, as C3b, Bb. This complex, like C4b, 2a in the classical pathway, is a C3 convertase (C3b; Bb); it is stabilized by the binding of another plasma protein, properdin. Thus, the alternative pathway used to be called the properdin pathway. The presence of a single molecule of C3b generates many molecules of C3b, Bb, resulting in a tremendous amplification. The C3 convertase (C3b, Bb) cleaves C3, thereby generating more molecules of C3b, which can combine with other molecules of factor B to give more molecules of cab, Bb, which can, in turn, cleave more molecules of C3. Therefore, the central feature of the alternative pathway is a positive feedback loop that amplifies the original recognition event. As in the classical pathway, attachment of many C3b molecules to the target cell will allow binding of C5 and its cleavage into C5a and C5b by the enzyme C3b, Bb, now referred to as C5 convertase.

Owing to the potential of this positive feedback loop to rapidly use up Factor B and C3, the positive feedback must be carefully regulated. There are two important regulatory proteins in plasma. The first protein, Factor H (formerly referred to as PIH), competes with Factor B for binding to C3b and also dissociates C3b, Bb into C3b and Bb. The second control protein, Factor I (formerly referred to as C3b in activator), cleaves C3b that is bound to Factor H or to a similar protein found on the surface of the host cell. The resulting cleaved C3b, termed C3b, can no longer form a C3 convertase. The action of these two control proteins prevents the consumption of Factor B and C3 in plasma; in addition, these two proteins in activate C3b, Bb on host cell surfaces. In contrast, surfaces of many target cells, such as bacteria and other microorganisms, protect C3b, Bb from in activation by Factors H and I. This protection allows the positive feedback loop to proceed on the surface of the target cell, leading to the activation of the pathway and subsequent cell death. In other words, the alternative pathway is activated by those substances that prevent the inactivation of the positive feedback loop enzyme C3b, Bb. A substance is therefore treated as "foreign" if it restricts the action of Factors H and I and allows the positive feedback loop to continue.

The chemical structures on surfaces of particles and cells responsible for activation or non-activation of the alternative pathway have not been identified. There is some evidence that carbohydrate moieties are involved,

particularly sialic acid. The alternative pathway protein(s) responsible for the recognition of these structures also remains to be determined. As pointed out earlier, the activation of the alternative pathway requires a C3b molecule bound to the surface of a target cell. An intriguing question is, "Where does the critical first cab molecule come from?". Although it can be provided by the C3 convertase of the classical pathway or by cleavage of C3 by plasmin and certain bacterial and other cellular proteases, the alternative pathway can generate this first C3b molecule without these proteases. The intramolecular thioester, which is highly reactive in nascent C3b and is responsible for the covalent attachment to targets, is also accessible in native C3 to water molecules. Thus, spontaneous hydrolysis of the thioester bond occurs constantly in plasma at a low rate. The C3 molecules in which the thioester bond has been hydrolyzed behave like C3b, although the C3a domain has not been removed. C3 with a hydrolyzed thioester is called C3 or C3b-like C3. It can bind Factor B and allow Factor D to activated Factor B, which results in formation of a fluid-phase C3 convertase, C3, Bb. This enzyme is continuously formed and produces C3b molecules that can randomly attach to cells. Although these C3b molecules will be rapidly inactivated on host cells by Factors H and I, they will start the positive feedback loop on foreign surfaces, as outlined previously. In other words, the alternative pathway is constantly activated at a low rate, but amplification with subsequent cell death occurs only on foreign particles [21].

With this consept, we tried to demonstrate visually by the immune-electrophoresis. The human serum was prepared after administrating *f-Black Turmeric* together with the sample with befor fermentation. Immuno-electrophoresis was setting up for 90 min, followed by incubating with anti-human whole serum and specific for C3 and Bf component. These specific anti-complement component serum were kindly supplied by Dr. Syunnosuke SAKAI, Cancer Research Institute of Kanazawa University, Japan.

4.3.2. Products of Complement Activation by FBT Possessing Biological Activity

Activation of either the alternative or the classical pathway results in the generation of many important peptides involved in inflammatory responses. The anaphylaxis increase of vascular permeability degranulation of mast cells and basophils with release of histamine Degranulation of eosinophils Aggregation of platelets opsonization of particles and solubilization of immune complexes with subsequent facilitation of phagocytosis Release of neutrophils from bone marrow resulting in leukocytosis Smooth muscle contraction Increase of vascular permeability Smooth muscle contraction Increase of vascular permeability Degranulation of mast cells and basophils with release of histamine Degranulation of eosinophils Aggregation of platelets Chemotaxis of basophils, eosinophils, neutrophils, and monocytes Release of hydrolytic enzymes from neutrophils Chemotaxis of neutrophils Release of hydrolytic enzymes from neutrophils Inhibition of migration and indulation of spreading of monocytes and anaphylatoxins C3a, C4a, and C5a are derived from the enzymatic cleavage of C3, C4, and C5 respectively. Historically, C3a and C5a were defined as factors derived from activated serum possessing spasmogenic activity. The anaphylatoxins are now recognized as having many additional biologic functions. Both C3a and C5a are known to induce the release of histamine from mast cells and basophils. As shown in the Figure anaphylatoxins cause smooth muscle contraction and induce the release of vasoactive amines, which cause an increase in vascular permeability.

The effect of C5a anaphylatoxin on neutrophils is of considerable importance in the inaammatory response. Not only can C5a induce neutrophil aggregation, but this anaphylatoxin appears to be the main chemotactic peptide generated by activation of either complement pathway. *In vitro*, nanomolar concentrations of C5a will induce the unidirectional movement of neutrophils. Other inflammatory cells, such as monocytes, eosinophils, basophils, and macrophages, have also been shown to exhibit a chemotactic response to C5a. The removal of the carboxy-terminal arginine from C5a by serum carboxy peptidase N, generating C5a-des-arg, inactivates the spasmogen, yet restoration of full chemotactic activity of C5a-des-are may occur in the presence of serum. Therefore, C5a-des-arg may also be responsible for *in vivo* neutrophil chemotactic activity.

As described earlier, the cleavage of C3 by either the alternative or the classical C3 convertases results in the production of two major split products, the C3a anaphylatoxin and cab. The larger C3b fragment can serve as an opsonin (promoter of phagocytosis) by binding to a target through the thioester mechanism. This renders the particle or cell immediately susceptible to ingestion by a variety of phagocytic cells that carry specific receptors for C3b.

Many recent observations point to additional roles for complement fragments in regulating the activity 9f cells of the immune system. These observations include the presence of receptors on lymphocytes for various complement proteins, including C3 split products and Factor H, affecting B- and T cell function. This is an important

area for future research [21] (**Figure 5**).

5. Discussion

Our investigation clarified how hemopoitic formula, also known as tonic agent, 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 FBT 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 FBT augmented lymphocyte production qualitatively than quantitatively. Moreover, FBT 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 FBT acted in macrophages in the same manner as *Mycobacterium tuberculosis* that had cell walls constructed of waxy substances which were famous for activating phagocytes in mammals [23]-[25].

In previous reports 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 FBT. By the following conditions. the excitement of sympathetic nerve system directly regulated granulocytes in number and function,while lymphocyte count was regulated by parasympathetic nerve system [11] [26]-[29]. 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

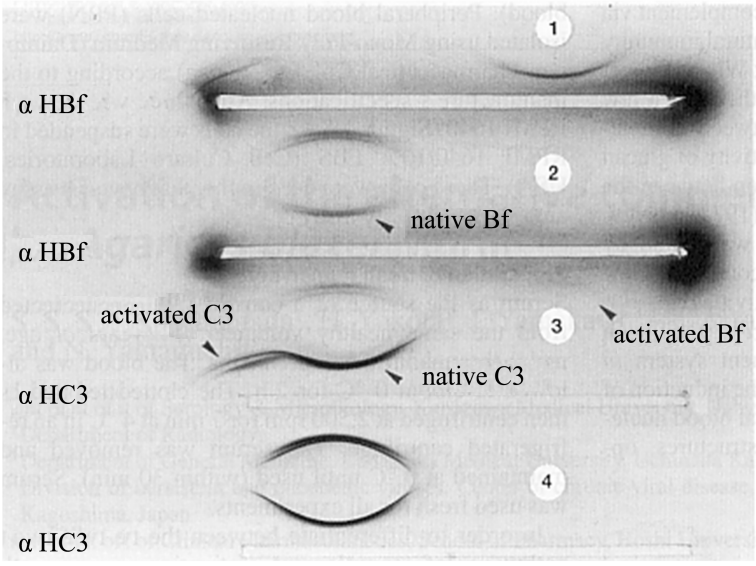

Figure 5. Immuno-electrophoretic demonstration of activated human complement components.

increased in the same subjects. In other words, the subjects dominated. The sympathetic nerve system could release stress, more over FBT could help these sympathetic neve system through the emotional hormones such as adrenalin and dopamine. 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 might influence cells throughout the body and might pass through every organ system, and hemopoitic formula stimulation might provide maximum benefits without side effects [30]-[35]. As an immunopotenciating agent, this FBT was an interesting material for regulate peripheral leukocyte number that suggesting one's constitution. FBT also regulated leukocyte subset, granulocyte and lymphocyte ratio concerning constitution, including QOL for all young to the senile.

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Abbreviations

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.
CBT: Conventional Black Turmeric before fermentation.
DM: Diabetes mellitus.
FBT: Fermented Black Turmeric, that had been depredated to micro fragment by Lactobacillus.
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.