

# *Lactobacilli* Enjoyed Fermented Herbs on to the Last Fragment and Regulated Leucocyte Subsets and Anti-Oxidative Activity

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## Abstract

Eighty sorts of herbs were fermented by *Lactobacilli* (*f-ESH*). This material was proved by as safe in animal safety experiment. This work was aimed to revise the traditional way of hot water extraction to the fermentation, in order to use up the original material and searching new activity as well. We tried to prove the new activity by fermentation for immune-competent cells in the host that administrated both of original remedy and the new fermented sample. In rodents, immune-compromised host was prepared with cancer chemotherapeutic agent. Our observations showed that the antigen stimulated animal increased the antibody producing cell for heterologous erythrocyte by compromised host by *f-ESH*. Moreover, by administration of *f-ESH* to immunosuppressed hosts, these samples regulated lymphocyte in number and functions, macrophage activities, and regulating anti-oxidative activity by phagocytic cells. The anti-oxidative assay was carried out *ex-vivo* system by peritoneal exudate cell that we propose as suitable system for evaluating anti-oxidative assay. With these evidences, the *original* COF only augmented the level of lymphocytes in number, while *f-ESH* regulated the ratio of granulocytes and lymphocyte. In clinical study with 20 healthy volunteers, granulocyte and lymphocyte ratio was also resulted as neutral in peripheral white blood cells, 1, 2 and 3 weeks after the administration of *f-ESH*. We discussed the significance and mechanism of cleaving complement components Fb by *f-ESH* in this text. Fb. The new cleavage of complement was directly evident by immune-electrophoretic method. In conclusion, this modification of food

materials introduced new style of food intake resulted in the significant regulation such as antibody producing cells and anti-oxidative activity for phagocytic cells comparing traditional processing by hot water extraction.

### Keywords

Wild Herbs, Fermentation, *Lactobacilli*, Lymphocyte, Granulocyte, Ant-Oxidative Activity, GABA, Complement, Alternative Complement Activation

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## 1. Introduction

About our defense system, the overwhelming problems of developing our dual system, the innate and inductive do not seem to guard or even prevent the development of one internal threat to survival. Moreover, every individual exposes to the risk of immunodeficiency in daily life with both internal and external factors. Complementary and alternative medicines (CAM) have achieved more and more attentions since they are able to cure many chronic illnesses, such as fatigue syndrome that plagues the developed world. The present study had concerned that typical styles of CAM, preparing special molecule for both digestive and easy to activated human complement component that regulate functions of leukocytes in human as well as animal immune system [1]-[7]. Dietary and fermented formula held promise as strong inducers of augmentation of acquired immunity. While the immune system is working against the local infection of pathogens, cytokine and immuno-competent cells react throughout the whole physical system in close connection to the brain, the endocrine and immune system [1] [2] [3] [4] [5]. In this report, we hypothesized that *f-ESH* might influence immuno-competent cells qualitatively and quantitatively *f-ESH* targeting lymphocytes based on the constitution dependent manner. COF had 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, metabolic disorder such as diabetes mellitus, malnutrition, extreme exhaustion, stress, aging and medical side effect in cancer [6] [7] [8] [9] [10]. So we have to select appropriate menu to regulate immune function through leukocyte storage. The menu has been summarized and listed as CAM: complementary and alternative medicine.

In this study, we tried to focus on the identity of *f-ESH* formula, comparing to another non-fermented decoction. The hot water extraction was traditionally succeeded to the present, wasting almost all the original material was discarded after use. With a series of trial, we tried to process the original material into small fragment by microbe and to find new molecule to affect our physical system as well as use up of the material for global sense of ecological system. The influence of *f-ESH* on leukocyte and/or lymphocyte subpopulations in human peripheral blood was also discussed. One of the main purposes of this work was

to revise the traditional way for preparation of remedy, from hot-water extraction to fermentation in order to use up the original material and searching new activity beside of hot water extraction. For the preparation of TCM remedy, it was succeeded as hot water extraction, wasting the almost all the original material as that of many tea extraction/preparation in all over the world. Moreover, some preliminary trial that concerned the new processing of the original material by degradation by acidophilic bacteria, fermentation.

## 2. Materials and Methods

### 2.1. Preparation of Wild Herbs and Fermented Herbs

Commercially available 80 sorts of wild herbs were prepared and extracted by 100 ml of hot water (98C) to 10 gr of grained roasted material. For 3 minutes. On the other hand, the fermentation was carried out by *Lactobacillus leuteria* for 5 days at 40C, and was repeated two times in the method described in M & M. After the centrifugation of 2000 xg for 10 min at room temperature and then, dried by heating as powder and served for *f-ESH*.

### 2.2. Fermentation and GABA Generation

Commercially available 80 sorts of wild herbs were prepared and extracted by 100 ml of hot water (98C) to 10 gr grained the roasted material. for 3 minutes. The fermentation was carried out by *Lactobacillus leuteria* for 5 days at 40C. Each ratio of powdered, *lactobacilli* and water was 100:50:850, prepared by ECHIGO YAKUSOU, Ltd. Niigata, Japan). After the centrifugation of 2000 xg for 10 min in a room temperature and supernatant was served for *f-ESH*. GABA: gamma amino acid butyric acid was evaluated by the test system [11] [12].

Followings were the method for quantifying  $\gamma$ -aminobutyric acid, which comprises the steps of producing reduced nicotinamide adenine di-nucleotide phosphate by using a specific aminotransferase and a dehydrogenase that needs to use oxidized nicotinamide adenine di-nucleotide phosphate as a coenzyme and deactivating the enzymes, thereby removing any amino acid having a similar structure to that of GABA and acting an electron carrier on NADPH produced in the aforementioned step in the presence of a tetrazolium salt that can produce a water-soluble formazan dye and measuring the water-soluble formazan dye described and reported in references [11] [12].

## 3. Animal Experiment

### 3.1. Single and Multiple Dose Toxicity

Seven-week-old ddY mice consisted with 7 individuals ( $21 \pm 1.5$  gr) 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 kept at animal room,  $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , 50% relative humidity. The selection of female mice was due to their gentle behavior in the

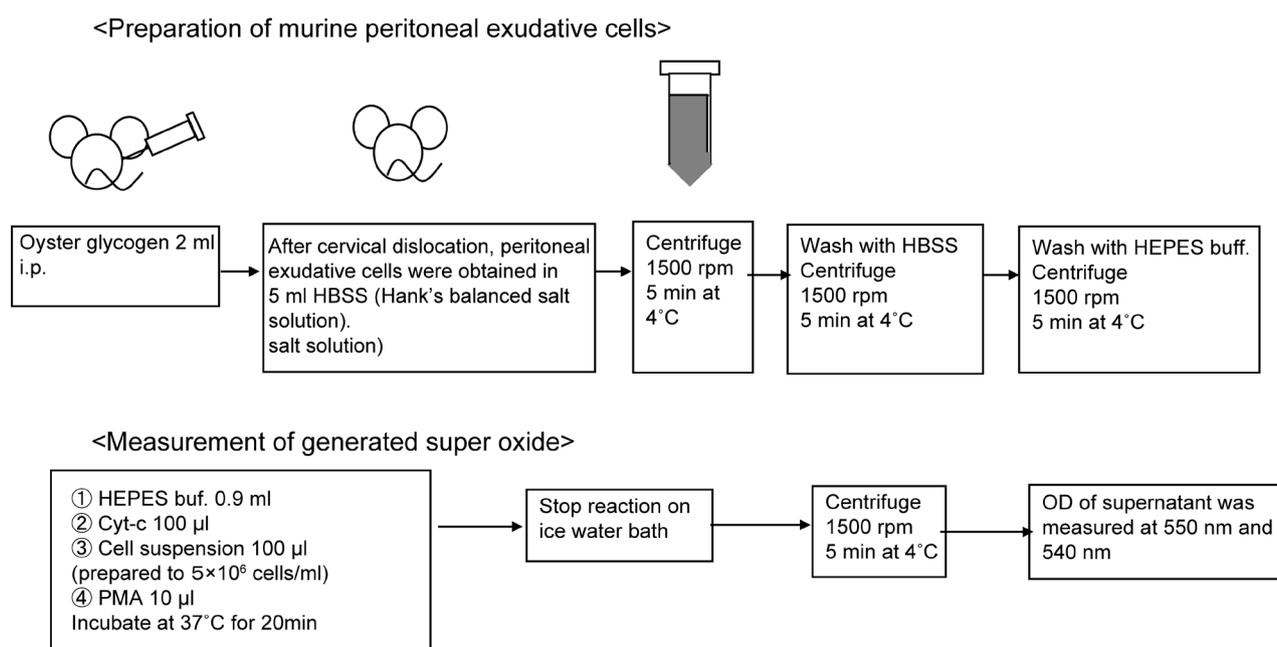
animal cage. Both conventional and fermented coffee were suspended in sterile and administered to 100 mg/day via stomach tube. Another food and water supply were freely administered. Mice were weighted at 0 - 7 days after administration, and clinical observations were made once a day. Necropsy was performed on all mice seven days after administration.

### 3.2. Animals Experiment for Preclinical Trial

Eight week-old female SPF 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  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and 60%.

### 3.3. Reagents and Preparation

About the fundamental medium for anti-oxidative activity, HEPES buffer (HEPES 17 mM, NaCl 120 mM, Glucose 5 mM, KCl 5 mM, CaCl<sub>2</sub> 1 mM, MgCl<sub>2</sub> 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 mouse 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  $120^{\circ}\text{C}$  for 20 min. This solution was used for intraperitoneal injection to mice in order to induce peripheral neutrophils into the abdominal cavity (**Figure 1**).



**Figure 1.** Experimental procedure for accessing Anti-oxidative activity of the murine Macrophage.

### 3.4. Assessing the Amount of Super Oxide Anion Generated by Mouse Macrophage

Each sample was orally inoculated 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 collected 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  $1 \times 10^6$  cells/ml of HEPES buffer. One hundred microliters of cytochrome-c and 10  $\mu$ l 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 optical density of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was estimated in the formula; increased absorbance at 550 nm ( $\Delta A_{550-540}$ )/ $19.1 \times 10^3$  (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 suppressed 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 inside the cytoplasm of phagocytic cells.

### 3.5. Statistical Analysis

Data are expressed as means  $\pm$  standard deviations. The differences between COF-treated and non-treated conditions were compared using a one-tailed analysis of variance. A *P* value  $< 0.05$  was regard to be statistically significant.

## 4. Results

### 4.1. Animal Test for *Fermented ESH*

#### 4.1.1. Single and Multiple Dose Toxicity Study for *Conv. ESH* and *f-ESH*

Seven female seven-week-old ddY mice, were used for the acute oral toxicity study. Mice were weighted at 0 - 7 days after administration, and clinical observations were made once a day. 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.

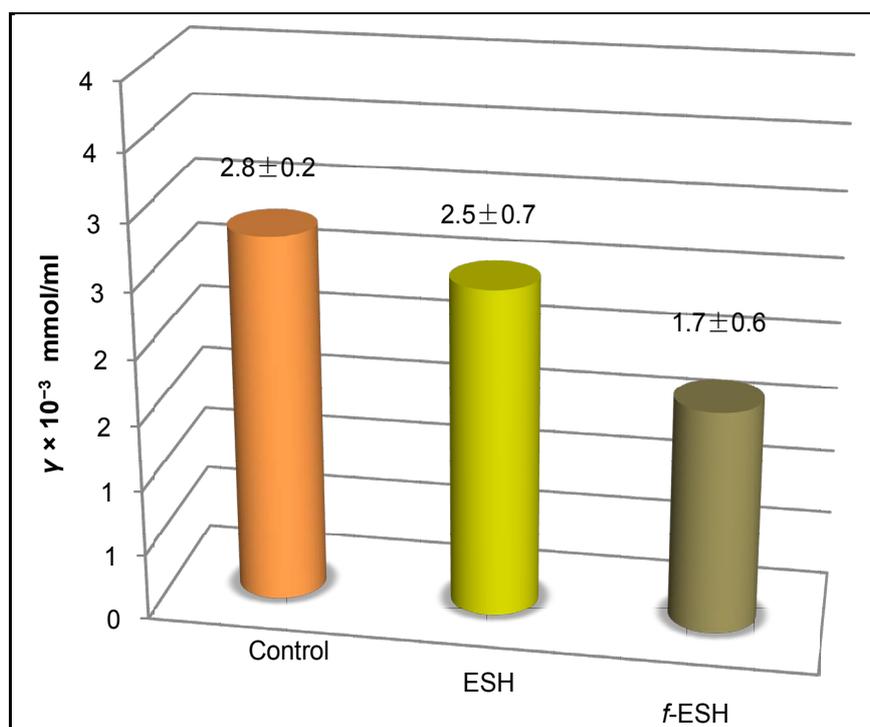
#### 4.1.2. Amount of Generated Super Oxide Anion Regulated by *f-ESH*

The amount of generated super oxide anion was calculated in the formula shown above. The generated super oxide anion after one week administration of *heat extracted coffee* and *fermented coffee* were  $2.3$  and  $1.6 \times 10^{-5}$  mmol/ml, respectively, whereas that was  $2.8 \times 10^{-5}$  mmol/ml in control group. All these samples, regulated heat extracted COF showed anti-oxidative activity to the control group ( $P < 0.05$ ) and fermented one showed more significant ( $P < 0.01$ ), decreased super oxide anion generation after administration for one week in mice (**Figure 2**).

## 5. Clinical Findings

### 5.1. Changes in Cell Number of Total Leukocyte and Subsets by Conv ESH & f-ESH

Leukocyte numbers have been counted one hour before and 15 days after the treatment of-ESH derivatives. 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-ESH. However, significant change was found in L-type group (Table 1).



**Figure 2.** Anti-oxidative activity by fermented coffee and conventional heat-extracted one.

**Table 1.** Constitution dependent regulation of leukocyte by *COF derivatives*.

	G type individual		L type individual	
	<i>f-ESH</i>		<i>f-ESH</i>	
	Before	After	Before	After
Total WBC (×10 <sup>3</sup> μl)	6.36	5.88	4.89	5.66
Lymphocyte (%)	25.8	32.6	44.7	36.8
Granulocyte (%)	68.4	66.3	54.8	61.3
Neutrophil (%)	63.5	52.6	42.6	57.3
Eosinophil (%)	1.7	2.3	2.5	3.5
Basophil (%)	0.7	0.8	0.6	0.7

## 5.2. 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 *f-ESH*; in G-type group, total number of leukocytes was down regulated by *f-ESH*. This was a result of the down regulation of major group of leukocyte, granulocyte (Table 2).

As for the G-type, no significant changes were found after the treatment of both COFs. In the L-type group, *f-ESH*, on the other hand, increased the total 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 coffee* but not by COF. 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 Coffee*, but not by *conventional Coffee* on day 7.

## 5.3. The Complement System; Another Stage for Focusing by Fragmented Polysaccharide by *f-ESH*

It was possible to focus on another important factor of immunological component, complement either classical and/or alternative pathway. These proteins are composed of at least 9 components. These proteins are famous for its acute arrangement 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

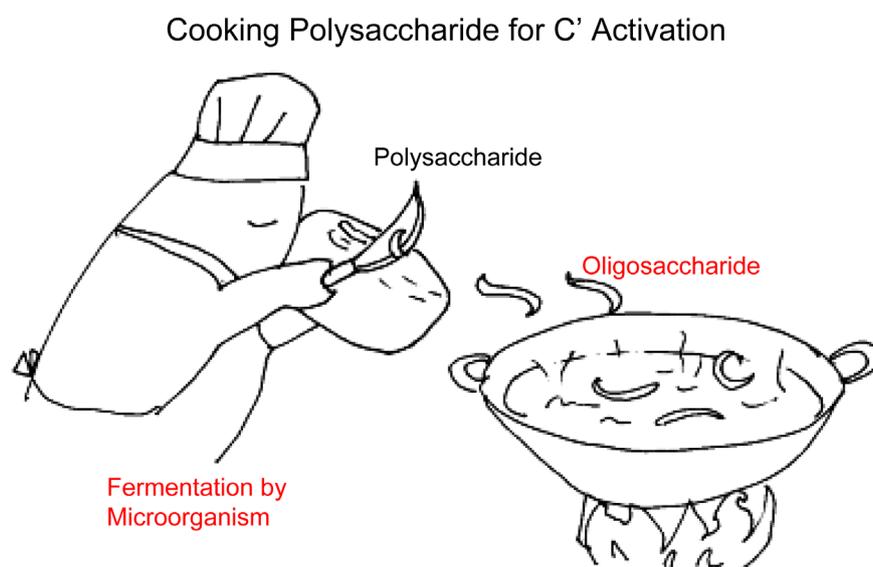
**Table 2.** Constitution dependent regulation of lymphocyte by FSH derivatives.

CD	G type individual		L type individual	
	<i>f-ESH</i>		<i>f-ESH</i>	
	Before (%)	After (%)	Before (%)	After (%)
CD2	64.6	71.7	62.43	61.84
CD4	18.44	29.64	31.65	30.678
CD8	37.86	43.65	26.77	27.98
CD11	73.89	66.75	62.75	69.54
CD14	0.04	0.07	0.06	0.08
CD16	67.55	59.86	54.24	47.64
CD19	8.77	8.98	8.66	7.88
CD56	1.34	1.46	1.48	2.79

pathway adjunct 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 [15] [16] (**Figure 3**).

#### 5.4. Pathways of Complement Activation and Complement Proteins by *f-ESH*

The complement elements can activate by two pathways: the classical and the alternative pathways. Both pathways lead to a common terminal channel correspond 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



**Figure 3.** Illustrative imaging for degradation of polysaccharide by microorganism.

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, C1r, and C1s. 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<sub>3</sub>) or Ig 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, C1r 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 C1r, a protease that subsequently cleaves the proenzyme C1s. 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 C1r and C1s. The final result is the generation of an enzymatically active component, C1s, 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 electrophoretic methods.

The enzymatic protein C1s has two physiologic substrates, C4 and C2. C4 is cleaved by C1s 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. C1s 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 C3b, 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 marked cells ("metastable binding site"). This property has recently 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 C3b, 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.

According 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 regulatory protein, Factor I (formerly referred to as C3b inactivator), 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 iC3b, can no longer form C3 convertase. The action of these two control proteins prevents the consumption of Factor B and C3 in plasma; in addition, these two proteins inactivate C3b, Bb on host cell surfaces. In contrast, surfaces of many target cells, such as bacteria and other microorganisms, protect C3b, Bb from inactivation 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 limits the action of Factors H and I and allows the positive feedback loop to succeed.

The chemical structures on surfaces of particles and cells responsible for activation or non-activation of the alternative pathway have been identified [22]. 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 C3b 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 activate 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 [17].

With this concept, we tried to demonstrate directly by immunoelectrophoresis. The human serum was prepared after administering f-Black Turmeric together with the sample with before fermentation. Immunoelectrophoresis was set up for 90 min, followed by incubating with anti-human whole serum and specific for C3 and Bf component. These specific anti-complement component sera were kindly supplied by Dr Syunosuke SAKAI, Cancer Research Institute of Kanazawa University, Japan (Figure 4).

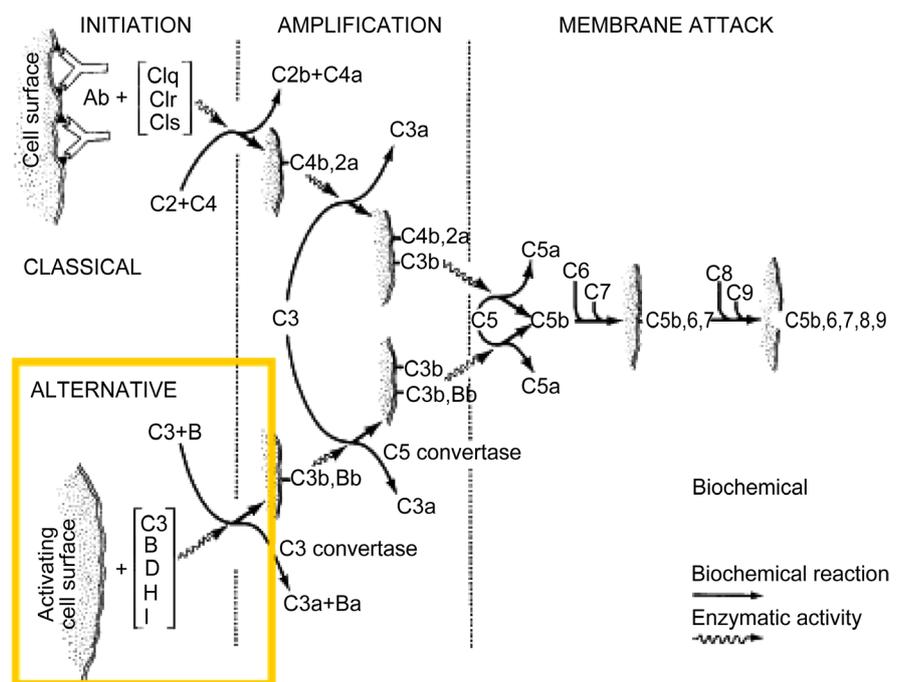
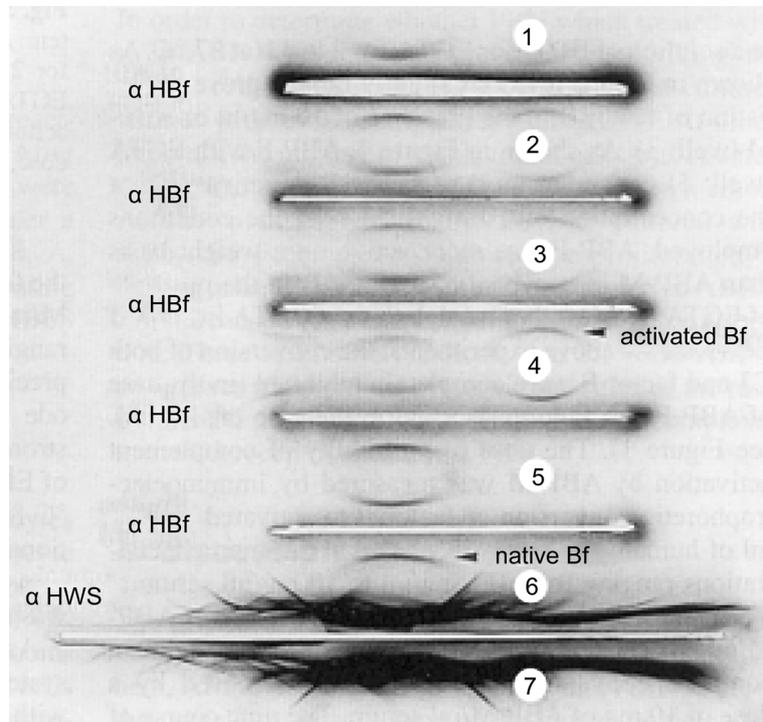


Figure 4. Diagrammatic representation of alternative complement component activation.

### 5.5. Direct Evidence of Complement Products Activation by *f-ESH* and Translation of Biological Activity

Activation of either the alternative or the classical pathway results in the generation of many key peptides involved in inflammatory condition. 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 modification 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 undulation 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 inflammatory 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*, nano-molar 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 carboxyl 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-desarg 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 of 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 [13]-[18] (Figure 5).



**Figure 5.** Immunoelectrophoretic demonstration of activated human complement components.

## 6. Discussion

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. We reported that about hot-spring hydrotherapy and acupuncture, and had proposed that immune system regulation is an important factor for evaluating CAM. 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 remain unclear. Further research regarding to the mechanism is necessary. The excitation of the sympathetic nervous system while lymphocyte count is increased by excitation of parasympathetic nervous system [19]-[25]. 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 FSH the sympathetic nerve could release stress, whereas the sympathetic activity of subjects who were dominated FSH. 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 hemophilic formula did not cause infection. Since the meridian may influence cells throughout the body and may pass through every organ system, hemophilic formula stimulation might provide maximum benefits without side effects [26]-[29]. As an immune-enhancer, hemophilic formula merits further investigation as a possible treatment for acquired immunodeficiency syndrome, chronic fatigue syndrome.

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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.

*f-ESH*: Fermented Coffee that had been depredated to micro fragment by Lactobacillus.

FCM: Flow Cytometry.

G-rich type: An individual that exhibit over 60% of granulocyte in peripheral blood, finding many in young gentleman.

L-rich type: An individual that exhibit over 40% of lymphocyte in peripheral blood, finding lot in ladies and senile.



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