

Fermented Ethnic Medicine (*Pueraria mirifica*) Designed by Lactobacillus Regulated Leukocyte Subsets via Activation of Complement Components

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Abstract

A plant fermentation was carried out by *Lactobacilli* against the Rhizome from *Pueraria milifica* (*f*-PMF). This material was evidenced by safe in animal toxic study. The main aim of this study was to revise the traditional way of hot water extraction to fermentation so as to use up the original material and finding new activity. We tried to show the new activity through phytoestrogen and immune-competent cells from the host that administrated either of original remedy and the new fermented sample, plus activated water SRE. In mice, compromised host was prepared by cancer chemotherapeutic agent (Mitomycin-C). After administration of *f*-PMF to immuno-suppressed animals, the effects by both samples were augmented by lymphocyte in number and functions, macrophage activities, anti-oxidative activity. However, the intense of effect was much more by fermented one but not by conventional one. The anti-oxidative assay was also carried out *ex-vivo* system by peritoneal macrophage that we proposed as suitable system for evaluating anti-oxidative assay. In our clinical study by 20 healthy volunteers, granulocyte and lymphocyte ratio was regulated as neutral in peripheral white blood cells, increasing one, two and three weeks after the administration of *f*-PMF. We have found the significant regulation of blood chemical factors that were important makers for the lifestyle-related diseases. The mechanism of augmentation by probing directory with immuno-electrophoretic method, generating new complement component, especially found by alternative pathway of complement. So we discussed the process concerning designed *f*-PMF molecule for activation of complement component and bound for the biological activity of each physical component. In a limited condition, fewer numbers of volunteers, the breast

size was tending to increase along with the administration time. Including these evidences, we discussed the possibility of this traditional ethnic medicine, originally found and spread in the highland area in Thailand and Myanmar.

Keywords

Family *Pueraria*, Fermentation, *Lactobacilli*, Cancer Chemotherapeutic Agent, Immune-Compromised Host, Lymphocyte, Granulocyte, Ant-Oxidative Activity, Complement, Alternative Complement Activation

1. Introduction

Recently, complementary and alternative medicines (CAM) have been condensed since they are able to cure many chronic diseases, such as chronic fatigue syndrome that plagues the developed society. The present team had reported 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]-[8]. Dietary and fermented formula held promise as powerful inducers of adoptive immune system. While the immune system attack against the local infection of pathogens, interleukins and immuno-competent cells reacted throughout the body in close linkage to the brain, the endocrine and immune system [9] [10]. In this study, we hypothesized that *f*-PMF might influence immuno-competent cells qualitatively and quantitatively *f*-PMF targeting lymphocytes based on the constitution dependent manner of the host. PMF 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. In this report, we sought to focus on the identity of *f*-PMF formula, comparing to another herbal medicine. The effect of *f*-PMF on leukocyte and/or lymphocyte in human peripheral blood was also discussed. The aim 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 instead of hot water extraction. For the preparation of TCM remedy had been succeeded as hot water extraction for a thousands of years, 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. The material used in this report was originated and traditionally selected as ethnic medicine for plant-estrogenic supplement for female at the highland area in Thailand and Myanmar [11] [12].

2. Materials and Methods

2.1. Fermentation of PMF and GABA Accumulation

Commercially available powdered *Pueraria mirifica* were purchased from Kohkandoh Pharmaceutical Co. Ltd., Tokyo). The fermentation was carried out by

Lactobacillus leuteria for 5 days at 37°C at the Jar Fermenta (Yamato Rika Kikai. Co. Ltd., Tokyo). Each ratio of powdered PMF, lactobacilli and water was 100:50:850. After the centrifugation of 4000 × g for 20 min and supernatant was served for fermented PMF. For the indirect evidence of degradation of PMF, GABA: gamma amino acid butyric acid was evaluated by the test system reported by following administration.

Followings were the method for quantifying γ -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 by the references [13] [14].

2.2. Special Solution in This Study

We employed two materials for employed as solution of the material.

One is regular purified distilled water (DW) and specially activated water; SRE (KAOU, Co. Ltd., Fukuoka, Japan).

2.3. Single Shot and Multiple Shot Toxicity Study

Ten female seven-week-old ddY mice (19 ± 2 gr), were used for the acute oral toxicity experiment. The selection of female mice was due to their gentle behavior in the animal cage and the material used for this report was the under the aim of post-menopausal use of human. The safety test was carried out in aim of Ethics of the Organization for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were kept at 24°C \pm 1°C, 50% relative humidity. Both conventional and *f*-PMF were suspended in sterile water and administered to mice in free supplemental system, calculating daily consumption around 100 mg/kg. 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 the administration.

2.4. Experimental Model for Bone Marrow Suppressed Immune-Suppressed 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, PMF extracts was administered orally at a dosage of 100 mg/kg/day for six consecutive days. Conventional PMF and fermented PMF were chosen as controls.

Recovery of Total Leukocytes and Its Subsets, Granulocyte & Lymphocyte Ratio

The bone marrow-suppressed mice were administered herbal decoction *f*-PMF 1

g/kg dairy for 5 days and after 1 week later, their blood were collected from their tail vein. Then, the number of leukocytes was counted in Bürker-Türk counting system.

2.5. Assessment of Anti-Oxidative Activity

2.5.1. 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 $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 60%.

2.5.2. 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.

2.6. Statistical Analysis

Data are expressed as means \pm standard deviations. The differences between PMF-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 PMF and *f*-PMF

Single and Multiple Dose Toxicity Study for Conventional PMF and Fermented PMF

Ten female seven-week-old ddY mice were used for the acute oral toxicity study. Both mice were weighted at 0 - 7 days after administration of each PMF derivative, 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 experimental group.

3.2. The Comparison of Generated Super Oxide Anion between the Fermented and Conventional PMF

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 proceeded 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 conventional PMF, *f*-PMF and *f*-PMF + SRE were 2.9 , 1.8 and 1.2×10^{-5} mmol/ml, respectively. All the fermented herbal medicine decreased super oxide anion generation in compare with their corresponding unfermented ones (Figure 1).

4. Clinical Trial

4.1. Changes in Cell Number of Total Leukocyte and Subsets Change by *f*-PMF

Leukocyte numbers have been counted one hour before and 1, 2 and 3 weeks after the treatment of fermented PMF. The cell number measured one hour before the administration was set as 100%. Relative percentage of cell number on the one, two and three weeks were followed up to three weeks after the trial in case of human. As a result of this inspection, leukocyte subsets and platelet were increased and significant group were shown in red in Table 1.

4.2. Detecting Biochemical Factors after Administration of PMF Derivatives

The volunteers were healthy subject, with no drastic change for the total number of leukocytes. However, we tried to check the regulative effect of PMF on the

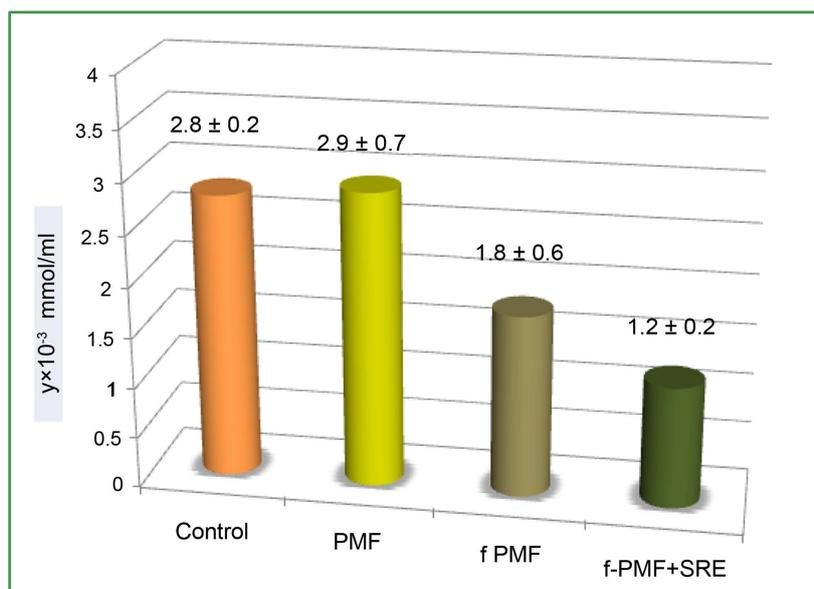


Figure 1. Anti-oxidative activity by fermented PMF and conventional one. The bone marrow-suppressed mice were administered PMF 1 g/kg dairy for 30 days, and one week later, their blood was withdrawn from their tail vein. The cell count of the peripheral blood is showed in the text. The Figure shows that the oxidative activity decreased to half of normal control after 5 mg/kg O-PMF and *f*-PMF administration [8].

Table 1. Hematological analysis of volunteer administered by *f*-PMF.

Parameter	Weeks after Trial		
	1 W	2 W	3 W
WBC ($10^9/L$) (%)	7.12 ± 1.15	7.09 ± 1.11	7.32 ± 1.62
RBC ($10^{12}/L$)	4.57 ± 0.52	4.58 ± 0.55	4.68 ± 0.45
Hemoglobin (g/L)	138.22 ± 17.14	137.78 ± 17.54	141.22 ± 14.81
HCT%	0.41 ± 0.05	0.41 ± 0.05	0.42 ± 0.04
MCV (fL)	89.31 ± 4.20	89.79 ± 4.85	89.00 ± 4.65
MCH (pg)	30.31 ± 2.04	29.77 ± 1.80	29.97 ± 1.70
MCHC (g/L)	338.84 ± 12.48	330.89 ± 6.58	336.78 ± 7.03
Platelet ($10^9/L$)	224.08 ± 37.66	221.23 ± 34.55	232.11 ± 35.33
PCT%	0.20 ± 0.05	0.19 ± 0.03	0.21 ± 0.05
PDW (fL)	16.23 ± 0.97	16.48 ± 0.45	16.36 ± 1.12
MPV (fL)	8.98 ± 1.22	8.47 ± 0.62	9.36 ± 1.21
Lymphocytes (%)	31.76 ± 7.65	31.54 ± 9.55	22.83 ± 7.45
Monocytes (%)	7.15 ± 1.66	7.06 ± 2.23	6.69 ± 1.81
Neutrophils (%)	58.43 ± 8.76	59.47 ± 7.54	56.22 ± 9.44
Eosinophils (%)	2.17 ± 1.67	2.02 ± 1.67	1.79 ± 1.44
Basophils (%)	0.46 ± 0.21	0.46 ± 0.30	0.38 ± 0.21
Lymphocytes ($10^9/L$)	2.23 ± 0.56	2.17 ± 0.45	2.52 ± 0.38
Monocytes ($10^9/L$)	0.49 ± 0.14	0.52 ± 0.13	0.47 ± 0.24
Neutrophils ($10^9/L$)	4.15 ± 1.19	4.25 ± 1.22	4.02 ± 1.43
Eosinophils ($10^9/L$)	0.14 ± 0.12	0.15 ± 0.21	0.14 ± 0.13
Basophils ($10^9/L$)	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01

The analysis of CD positive cells by FCM was measured by gating in the lymphocytes region on the scattered gram. Figure shows an example analysis. Nonspecific reaction of the PE fluorescence was found in the isotype control.

biochemical factors. Analysis that mixed both groups together showed no significant differences in TG value. Not significant but some trend was seen in high-dense cholesterol and low-dense-cholesterol. Each of factors move to the better condition and indicated in green in **Table 2**. As a results of total effect of this material *f*-PMF increased the bust size of volunteer ladies of senile.

The evident was shown that many leukocyte subsets regulated by *f*-PMF, we tried to check immune-globulin level in the *f*-PMF, administered volunteer. Manu of immunoglobulin level were increased after administrating *f*-PMF. However, only IgE was down regulated by this remedy (**Table 3**).

4.3. The Complement System-Another Stage of Evidence for Focusing by Fragmented Polysaccharide, *f*-PMF

We would like to focus on another important factor of immunological component, COMPLEMENT. These component proteins are composed of at least 9 components. These component proteins are famous for its defensive activity against infections organisms as in the defense immunity. However, we had found

Table 2. Biochemical analysis of volunteer analysis administered by *f*-PMF.

Parameter	Weeks after Trial		
	1 w	2 w	3 w
ALT (U/L)	21.81 ± 2.18	22.16 ± 2.22	20.36 ± 3.22
AST (U/L)	19.23 ± 1.45	20.46 ± 1.31	18.16 ± 1.19
ALT/AST	1.11 ± 0.15	1.08 ± 0.17	1.12 ± 0.11
GGT (U/L)	37.21 ± 4.34	32.61 ± 5.13	37.89 ± 3.43
ALP (U/L)	120 ± 12.11	115 ± 9.21	121 ± 14.45
TBIL (umol/L)	13.20 ± 12.11	13.12 ± 12.11	13.27 ± 12.11
DBI (umol/L)	4.95 ± 0.29	4.05 ± 1.39	4.73 ± 0.43
IBIL	8.25 ± 1.41	8.08 ± 1.04	8.26 ± 1.21
TP (g/L)	75.34 ± 12.17	76.34 ± 9.78	79.21 ± 10.23
ALB (g/L)	52.90 ± 8.27	54.21 ± 7.32	55.12 ± 9.21
GLB (g/L)	33.31 ± 3.27	34.87 ± 4.23	34.45 ± 5.54
ALB/GLB (g/L)	1.43 ± 0.21	1.53 ± 0.24	1.64 ± 0.17
Urea (nmol/L)	5.36 ± 0.68	5.57 ± 0.45	5.59 ± 0.71
Crea (umol/L)	69.12 ± 9.78	61.62 ± 8.81	60.51 ± 6.96
UA (umol/L)	116.12 ± 9.91	114.61 ± 12.89	111.83 ± 13.91
GLU (mmol/L)	5.57 ± 1.06	5.20 ± 0.89	5.02 ± 1.20
TG (mmol/L)	1.87 ± 0.19	1.58 ± 0.28	1.06 ± 0.34
CHO (mmol/L)	4.12 ± 0.39	4.09 ± 0.22	4.02 ± 0.15
HDL-C (mmol/L)	1.09 ± 0.27	1.17 ± 0.39	1.39 ± 0.17
LDL-C (mmol/L)	2.45 ± 0.21	1.85 ± 0.13	1.45 ± 0.36
K (mmol/L)	4.56 ± 0.22	4.62 ± 0.43	4.79 ± 0.24
Na (mmol/L)	138.12 ± 3.17	136.51 ± 5.01	138.03 ± 4.19
Chloride (mmol/L)	95.03 ± 1.31	97.67 ± 0.72	98.57 ± 2.12
Calcium (mmol/L)	2.21 ± 0.04	2.41 ± 0.02	2.37 ± 0.04
Breast Size	100%	104%	108%

The analysis of β_2 -AR receptor expressing cells and CD positive cells by FCM was measured. By gating in the lymphocytes region on the scattered gram, figure shows an example analysis.

Table 3. Immunoglobulin class analysis of volunteer analysis administered by *f*-PMF.

Parameter	Weeks after Trial		
	1 w	2 w	3 w
IgA (g/L)	2.25 ± 0.87	1.99 ± 0.79	2.47 ± 1.21
IgG (g/L)	12.92 ± 2.18	11.66 ± 2.06	12.41 ± 2.23
IgM (g/L)	1.40 ± 0.64	1.33 ± 0.62	1.54 ± 0.42
IgE (g/L)	71.96 ± 66.73	74.85 ± 47.75	55.66 ± 58.56

Time interval of blood sampling between before and after hot-spring hydrotherapy was approximately 24 hours. Measurements of the total and differential leukocyte counts and 3 catecholamines levels in the peripheral blood.

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) [15] [16].

4.3.1. Pathways of Complement Activation and Complement Proteins by *f*-PMF

The complement component can activate by two different pathways: the classical and the alternative pathways. Both pathways guide to a common terminal pathway result to as the pathway of membrane attack complex. Twenty plasma proteins are now known to be constituents of these pathways. These components can be divided into functional proteins, which represent the elements of the various pathways, and regulatory proteins, which exhibit each biological function. The serum level of the proteins in normal human varies a broad range. They are synthesized in the liver, spleen 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 complex. Following an initial recognition document, which leads to initiation of the pathway, an integration 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 attacked. The recognition unit for the classical pathway, C1, is composed of three important proteins, Clq,Clr, and C1s. The initiation of this pathway of complement typically involves the reaction of antibody plus antigen, which may be destroyed or on the surface of a target cell. This antigen-antibody complex allows the binding of Clq to two or more Fc regions of certain IgG subclasses (IgG1, IgG2, IgG,) or Ig activators of the classical pathway. The fine structure of Clq has been demonstrated by scanning 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 component. In addition to bind 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 result to cleave the proenzyme C1s. Thus, the binding

of Clq to an immunoglobulin in complex with the antigen represents the recognition unit of the classical pathway, resulting in the activation of C1r and C1s. The final event 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 unit. The other integration pathway, polysaccharide molecule also hits the complement component that is the main purpose of this report, inducing biological activities by fermented *f*-PMF. Therefore, some polysaccharide molecule hit the complement component in the manner of alternative pathway. Thus, *f*-PMF 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 target 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 gammacarbonyl 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 identical 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 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 iC3b, can no longer form C5 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 results in the positive feedback loop to proceed on the surface of the target cell, resulting in the activation of the pathway and subsequent cell death. With this mechanism, the alternative pathway is activated by those substances that prevent the inactivation of the positive feedback loop enzyme C3b, Bb. A substance is therefore recognized as “foreign” if it restricts the action of Factors H and I and allows the positive feedback integration to continue. The chemical structures on surfaces of target 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 mentioned before, the activation of the alternative pathway requires a C3b molecule bound to the surface of a target cell. An intriguing question is, “What does the critical first C3b molecule kick off?” Although it can be presented 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 bonding 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. Instead, 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 the immune electro-

phoresis. The human serum was prepared after administrating fermented *Pueraria milifica* together with the sample with before 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 [8].

4.3.2. Products of Complement Activation by PMF, Exhibiting 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 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 [8] (Figure 2).

5. Discussion

Our investigation clarified how *f*-PMF affect the physiological factor, especially for female menopausal disorder and also known as tonic agent, influences the immune system (e.g. leukocyte, granulocyte and lymphocyte subsets in particular).

We quantified blood biochemical factors and find many advance compare than that of non-fermented PMF. 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 PMF quantitatively and qualitatively regulated leukocytes, granulocytes, lymphocytes and their subsets. With many merit for increasing high-dense cholesterol and decreasing low-dense one, we

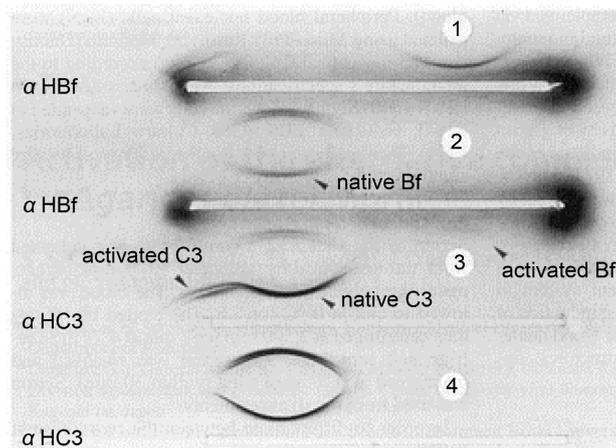


Figure 2. Immuno-electrophoretic demonstration of activated human complement. The human serum was prepared after administering *f*-PMF together with the sample with before fermentation. Immuno-electrophoresis was setting up for 90 min, followed by incubating with anti-human whole serum and specific for C3 and Bf component. These specific anti complement component serum were kindly supplied by Dr Syunnosuke SAKAI, Cancer Research Institute of Kanazawa University, Japan. Agar gel was dried and dyed by Trojan-Blue dye after de-saltation for three days [8].

also observed that the size up of bust o the volunteer. We got not enough dater for statistical significant, for the minimum number of the head. Together with sexual hormone such as progesterone, *f*-PMF augmented lymphocyte production qualitatively than quantitatively. Consequently, these data further demonstrated that PMF acted in the host as hormone supporting agent necessary for post menopausal ladies [11] [17] [18]. In previous reports about hot-spring hydrotherapy and acupuncture, we have proposed that immune system regulation is an important factor for evaluating CAM. Since other substances, such as endotoxin and waxy substances from *Mycobacterium tuberculosis*, similar to propolis, are 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 remain unclear. Further research regarding to the mechanism is necessary. 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 PMF the sympathetic nerve could release stress, whereas the sympathetic activity of subjects who were dominated PMF. 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 hemopoietic formula or not, the subjects were followed up for 8 days after the last administration of hemopoietic 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. As an immune-enhancer, hemophilic 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.

Conflict of Interest

We declared that there was no conflict of interest in this study.

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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.

DM: Diabetes mellitus.

f-PMF: Fermented *Pueraria milifîca* that had been depredated to micro fragment by Lactobacillus.

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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