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Regulative Effect for Granulocyte/Lymphocyte Ratio by Malted Rice through Complement Activation in Human Peripheral Blood

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Abstract

Background: A Life-related diseases had been claimed to regulated by finding some alternative medicinal process.

Objective: The purpose of this study was to find cool method to control the bias from life-related disease through granulocyte-lymphocyte ratio in human.

Method: A malted rice were prepared by Rice Yeast (MRY). This products were exhibited by safe in animal safety test. The trial was made to investigate a recovery of immune-competent cells both in granulocyte and lymphocyte ratio and CD positive lymphocyte subpopulations together with the amount of emotional hormone adrenalin and dopamine.

Result: Our results in animal model showed that MRY was the safe for the animal safety test. Four weeks after the administration, granulocyte and lymphocyte ratio was regulated as modulate and neutral. The emotional hormone adrenaline and dopamine level in serum also regulated neutral that dose dependent and reversible manner. The mechanism of this regulation had triggered by starting complement activation by absorbed MRY fragments through intestinal wall to the lymph.

Conclusion: We proposed an idea that MRY exhibited remedy effect for the regulation of immune competent cells via controlling complement component. The mechanism of activation was proved by activating alternative pathway of complement by the technic, immune-electrophoresis.

Keywords: Yeast; Fermentation; Molted rice; Granulocyte; Lymphocyte; Leucocyte subset ratio; Adrenalin; Dopamine; Alternative complement activation

Abbreviation: **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; **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; **MAC:** Membrane attack complex, the final complexed molecule for activating complement cascade, an important molecule for attack target cell such as invaded microorganisms; **MRY:** Rice koji mold (*Aspergillus oryzae*, *Saccharomycopsis fibuligera*).

Introduction

About defense system of vertebrate, the complexed problems of developing our dual system, the natural and acquirement do not seem to develop or even defensive the maturation of one internal event for survival. However, all the creature suffer from the risk of immune-suppressive in daily struggle with both internal and/or externals events, such personal habitat of individual, such in lifestyle related medical-failure [1]. From a decade, complementary and alternative medicines (CAM) have interested more and more attractive since they are able to treat many life-style related diseses, such as Diabetes mellitus, fatigue syndrome that spurred in the recent modern circumstances. This study had reported that representative styles of CAM, preparing special molecule for both digestive and easy to augmented human complement elements that neutralized the activities of leukocytes in lymph of adjusting immune system [2]. We had been reported and proposed that the health promoting supplement could work after digested the polysaccharide to downsizing fragment they activate complement element. This was particularly were part of the second complement pathway. Dietary Supplements and rice malt derivatives suggested as potent agent food supplement for regulating a natural immunity through the second complement pathway. These process were nearly resemble of the complement activating process different from invasive of infectios agent where the immune complex and/or toxic polysaccharide molecule such as lipopolysaccharide hutted as element of complement. Yet the immune system is working for the local invasion of infectious agent, macrophage worked out through the immune system related to the endocrine and nervous system In this study, it was supported hypothesized that MRY might activated on immuno-competent cells qualitatively and quantitatively, augmented lymphocytes according to the one'sconstitution.. MRY had been employed as heath promoting and the suggestion has little been made on the characteristics of the levels of leukocyte subset, such as granulocytes and lymphocytes ratio. In this report, it may also proposed to center on the identification of MRY active molecule, relating to another commercial material that was the first line of CAM. The activity of MRY on its ratio to leukocyte and/or lymphocyte subpopulations was also interested. The overall system of MRY to the phagocyte is also discussed in related to the complement

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activation especially the second complement pathway. This process was find to prepare repeated saccharide molecule but not single sugar molecule that enable to activate the complement pathway [3].

Materials and Method

Preparation of specimen for this study

Commercially available Rice koji mold (*Aspergillus oryzae, Saccharomycopsis fibuligera*) were prepared and daily administered 10 gr/day of MRY for 30 days.

Animal experiment

Ten female eight-week-old ddY mice, were severed for the acute oral toxicity observation. The experiment systems were carried out according to Ethics of the Organization for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were kept at 24 ± 1°C, 50% relative humidity in a SPF conditioned system.

Human trial

Harmonization had been issued, (ICH)/WHO Good Clinical Practice standards (GCP) including certification by an external audit. The trial protocol has been approved by according to the Research Ethical Committee of Kanazawa Medical University. Ten healthy volunteers aged from 21 to 70 years for both sex were recruited and were administered MRY for 30 days. After informed and consented to this trial, Fifteen milliliters of blood were drawn from the forearm vein one hour before the first administration of MRY and 30 days after the last MRY administration (day 30). All volunteers provided informed consent prior to participation for this trial. This study was approved by Ethics Committee of Kanazawa Medical University (Figure 1).

Leukocyte Counts Test in Peripheral Blood for MRY

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

Leukocyte Subset Analyses for MRY

The assessments including a total number of leukocytes was ordered to count with blood chemical test for the medical diagnosis of public institution (Ishikawa Preventive Medicine Association, Ishikawa, Japan). In the differential counting, 200 cells were counted on a May-Grunewald-Gimsa stained slide, and percentages of lymphocytes and granulocytes were determined [4-6].

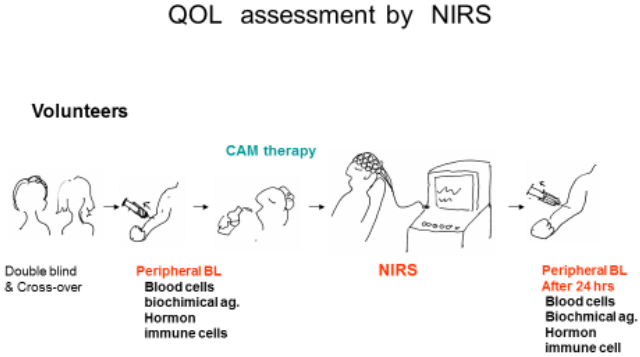

Figure 1: Research Design for this report, 10 volunteers were informed and consented by Ethics Committee of Kanazawa Medical University. Peripheral Blood were collected at the same time zone before and after the trial.

Lymphocytes and Lymphocyte Subset Analysis

The whole blood obtained from the subjects was washed twice with PBS (phosphate buffered saline, pH 7.2). The suspensions were treated with fluorescent mo-noclonal antibodies (FITC-conjugated anti-human CD2, CD4, CD8, CD16, CD19 and CD56) separately. After 30 minutes of staining at 4 degrees centigrade, the cells were analyzed by a FACScan (Becton Dickinson Co. Ltd. U.S.A.).

Cytokine Containing Cell Analysis

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 degrees centigrade. After that, the cell suspensions were stained using the monoclonal antibodies of PE-IL-4, FITC-IFN-γ and FITC-IL-1β, respectively. Then they were analyzed by the FACScan (Becton Dickinson Co. Ltd. U.S.A.). The antibodies and reagents used in the entire test were purchased from Becton Dickinson Immunocytometry system (U.S.A.).

Hormonal Level in Peripheral Blood

We ordered on the laboratory of Ishikawa Prefecture Preventive Medicine Association about the total and differential leukocyte counts and the levels of 3 catecholamines (adrenaline, noradrenaline, and dopamine) in the peripheral blood from the subjects. The total and differential leukocyte counts were measured by the automated hematology analyzer XE-2100 (Sysmex, Inc., Kobe, Japan). The levels of catecholamines were measured by high performance liquid chromatography (HPLC) system (Tosoh Co. and Hitachi High-Technologies Co.). As we here took an overview about the today's QOL assessment for the medicine in the east and the west, I think it is high time this medicine should be standardized uniformly and Japan could play an important role in this task [7-9].

Statistical Analysis

Data were analyzed employing the 2002-2003 SAS Version 9.1 software (SAS Institute Inc. Cary. NC, USA) represented as means ± standard deviations. The differences between MRY-treated and non-treated control were compared using a one-tailed analysis of variance. A P value <0.05 was regarded to be statistically significant.

Results

Animal safety test for MRY

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.

MRY 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.

Clinical Findings

Changes in cell number of total Leukocyte and Leukocyte subsets by MRY

Ten participants were finally selected in this study (age 63 ± 7.8).

They were all healthy volunteers to make informed and written consent of this trial. Leukocyte numbers have been counted one hour before and 30 days after the treatment of conventional or MRY. We recorded the cell number that were obtained bone hour before starting the trial was 100%. After 30 day of trial, the cell number were counted again and expressed in number and relative % in the Table 1.

Dividing subjects into two groups, G-type and L-type according to granulocyte and Lymphocyte proportion

The volunteer were healthy normal 10 individuals. A total number of leukocyte was significantly change after the administration of MRY. About the leukocyte subset ratio, the relative proportion of leucocyte subset, granulocyte and lymphocyte were changed significantly before and after the trial [10-13]. To further characterization to the influence of MRY, we separated 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 derived to down-regulated on day 30, accompanied by an increase in granulocyte numbers compared to control by MRY. On the other hand, the granulocyte counts of G type group indicated to decrease on day 30. The decrease of granulocyte count was augmented by MRY (Table 2).

Adrenalin/Dopamine also affect constitution

Each volunteer was prepared their blood before start to administered after informed consented to the experimental purpose by written Ethics of the Committee in Kanazawa Medical University. Beside the advance of VAS: visual analog scale in our investigation, we measured the total number of leukocyte and two major subsets, granulocyte and lymphocytes regulated before and after administration MRY. We tried to express the effect of peripheral total leukocyte number by individual level of change and plot in the x-axis as in each age. As was in Figure 2, the groups was separated in to two, up-regulated individuals and down-regulated one. The correlation of change was expressed as a linear function. Figure 2 was ideal change of effect with hot spring hydrotherapy, showing the best inclination. The results showed that these subsets could reflect the number and function of immuno-competent cells. For example, in an individual with a low granulocyte number, the number increased after treatment, while it decreased in another individual with a higher cell number. We sampled peripheral blood from the 10 volunteers before and after administration of MRY, at the same O'clock on the day, with the respect of circadian rhythm of leukocyte [14]. These subjects participated in this study after giving their informed consent. Measurements of the total and differential leukocyte counts and 3 catecholamines levels in the peripheral blood [15].

We ordered on the laboratory of Ishikawa Prefecture Preventive Medicine Association about the total and differential leukocyte counts and the levels of 3 catecholamines (adrenaline, noradrenaline, and dopamine) in the peripheral blood from the subjects. The total and differential leukocyte counts were measured by the automated hematology analyzer XE-2100 (Sysmex, Inc., Kobe, Japan). The levels of catecholamines were measured by high performance liquid chromatography (HPLC) system (Tosoh Co. and Hitachi High-Technologies Co. As we here took an overview about the today's QOL assessment for the medicine in the east and the west, I think it is high time this medicine should be standardized uniformly and Japan could play an important role in this task.

	G type individual		L type individual	
	MRY		*MRY*	
	Before	**After**	**Before**	**After**
Total WBC (x 10^3 µl)	6.44	5.78*1	3.47	5.65*1
Lymphocyte (%)	25.6	26.8	43.6	38.7*1
Granulocyte (%)	67.5	68.5	54.8	57.5*2
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
*1 P<**0.05** *2 P<**0.01**				

Table 1: Constitution dependent regulation of leukocyte by *Rice Yeast,* Volunteer were divide according to their constitution base on their granulocyte/Lymphocyte Ratio. The data represented the value obtained 30 days after MRY administration.

CD	G type individual		L type individual	
	MRY		*MRY*	
	Before (%)	**After (%)**	**Before (%)**	**After (%)**
CD2	66.6	76.76*1	60.43	77.65*1
CD4	19.54	28.44*1	31.43	45.67*2
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*1	54.24	46.67*1
CD19	8.45	8.21	8.41	7.95
CD56	1.57	1.88	1.78	2.87
*1 P<**0.05** *2 P<**0.01**				

Table 2: Constitution dependent regulation of leukocyte by *Rice Yeast,* Volunteer were divide according to their constitution base on their Lymphocyte Subpopulation expressed as CD positive Cell. The data represented the value obtained 30 days after MRY administration.

Granulocyte/Lymphocyte ratio Reveals Constitution

Traditionally, each heat therapy has its own character and efficacy for various complaints. Through the years, each water source was evaluated for its specific properties and with the advent of better transportation in our mountainous land, even remote springs in the mountains were visited for their specific medicinal effect.

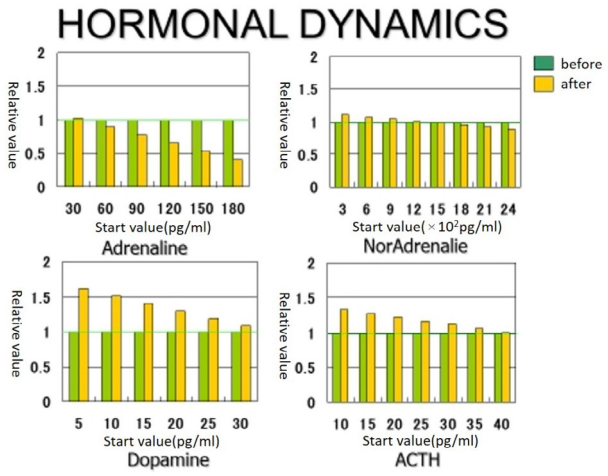

Figure 2: Hormonal Dynamics after Administration of MRY; The evaluation was ordered the laboratory of Ishikawa Prefecture Preventive Medicine Association about the total and differential leukocyte counts and the levels of 3 catecholamines (adrenaline, noradrenaline, and dopamine) in the peripheral blood from the subjects. The total and differential leukocyte counts were measured by the automated hematology analyzer XE-2100 (Sysmex, Inc., Kobe, Japan). The levels of catecholamines were measured by high performance liquid chromatography (HPLC) system (Tosoh Co. and Hitachi High-Technologies Co.) adrenaline (P<0.01), dopamine (P<0.01).

The Complement System-Another Step for Exhibition of Effect by Fragmented Polysaccharide in Lymph, MRY

We would like to check on another important factor of defense component factor, Complement. These protein are composed by at least 9 components. These protein are famous for its protective activity against infectious bacteria in the defense immunity. However, we had found that the complement had worked when we introduced fragmented/fermented polysaccharide, MRY, as complement activator, so called alternative pathway developed to Alternative Medicine. In this section, we would like to show the 1) what was the starting material in MRY, 2) which pathway of complement did kick out for activation, 3) what kind of physical activity was augmented as a result of this process. So in this report we would like to exhibit the character of complement and activated mechanism that lead to the augmentation of all the physical events through the activation of complement receptor positive structure cells. After generating complement component, various biological phenomenon were followed by activation, such as capillary swelling and scarlet phenomenon. These documented events were so called allergic processes. Activation of alternative of complement system results in a cascade of relation of these proteins, leading to the generation of products that have important biological functions such as swelling and scarlet phenomenon and that constitute an important humoral activating system involved in inflammatory site of the peripheral. We expected that the fragmented polysaccharide other than mono sugars, gently hit this alternative processes, leading to the total augmentation of physical activities. First, involvement of particles, such as bacteria or immune complexes, with certain parts of complement facilitates the digestion of the particle by macrophage etc., phagocytic cells (opsonic function of complement). Second, the activation event resulted many fission products of complement proteins for which specific receptors expressed on a variety of inflammatory cells, such as macrophage, neutrophil, lymphocytes, and other cells. Binding of these complement-derived elements to such receptors results in biologic activities such as chemotaxis and hormone-like activation of cellular activities.

The Specific Pattern of Complement Cascade and Complement Proteins by MRY

The two famous pathway process were known to drive to make final complement complex MAC: membrane attack complex. The original pathway was known to classical one and alternative one. The complement elements could move by two separate processes: the classical and the alternative pathways. In this report, imported fragment of saccharides plaied an important role as alternative pathway of complement. Both pathways guide to a common terminal referred to as the pathway of membrane attack complex MAC. Both the classical and the alternative complement pathways can be cascaded into various component units: initiation, amplification, and membrane attack complex. The initial recognition molecule, Ag-Ab complex and/or fragmented saccharide which leads to start of the complement processes, an integrative steps result to that involves the activation of relevant enzyme and the reuse of additional elements. These are followed by an end phase of membrane attack complex: MAC that the target cell destroy and next to autophagy phenomenon. The recognition unit for the classical pathway, The first component C1, is consisted of three independent elements, Clq, Clr, and Cls which derived from complement recognition element. The initiation of this pathfinder of complement characteristically drive the reaction of Ag-Ab complex, which may be conjugated or on the surface of a target cell. Thus the initiation of complement activation are activated by the binding of antigen-antibody complex in the classical pathway of complement activation. This antigen-antibody complex results the binding of Clq to many Fc regions of certain IgG subclasses (IgG1, IgG2, IgG,) or IgM. The visualization employing higher magnification by ultra-macroscopic technic recognized us to consist of six subunits similar to a component of six pedals relevant to the component of Clq. The other hit pathway, polysaccharide molecule, such as MRY also activated the complement component, especially alternative pathway. On the other side, some polysaccharide fragment hit the complement element in the manner of alternative pathway. Thus, MRY element hit human complement element and indicated by immune electrophoretic visual precipitated methods. In our lymphoid fluid, the gentle processing of component of Clq are occur instead vigorous movement of infection by the microorganisms [16-20]. Thus, naturally occurs consecutively in plasma at a low rate. The main structure of C3, C3a keep an initial structure, after thioester bond has been hydrolyzed The C3 molecules in which the thioester bond has been hydrolyzed just like C3b, but the C3a domain has not been diapered. We could visualized by immune-electrophoresis technic, C3 with a hydrolyzed thioester is named C3 or C3b-like C3 (Figure 3). This alternaive complement activation could be seen dose dependent manner in the recipient of MRY. It can conjugate Factor B and allow Factor D then cleave to Factor B, which develop in construction of a fluid-phase C3 convertase, C3. This enzyme is consecutively interact and compose c3b molecules that can randomly attract to cells. Therefore these C3b molecules will be promptly changed by the target cells by Factors H and I, the complement component will initiate the positive feedback construct on target surfaces, as indicated previously. On the other side, the alternative pathway is constantly activated at a low concentration of the starting material such as MRY, but amplification with subsequent cell death induced only on foreign particles such recently known as autophagy [21,22].

In this regards, we tried to prove visually by the immune-electrophoresis as immune-precipitation method. The human serum was collected 3 hours after oral administrating MRY. Incubation

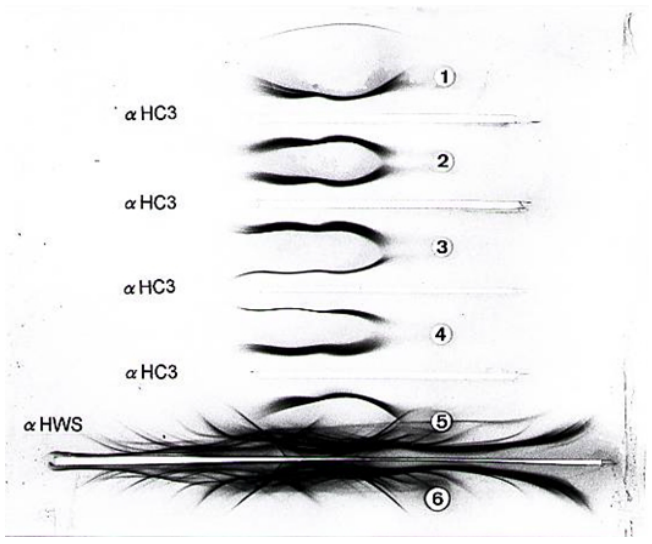

Figure 3a: Immuno-electrophoresis was performed in the agar gel. Figure show the results demonstrating activated in human C3 fragment.
1) Human serum that administered 15 gr of MRY/day.
2) 10 gr/day.
3) 5 gr/day.
4) Inactivated serum.
5,6) were MRY–free human serum

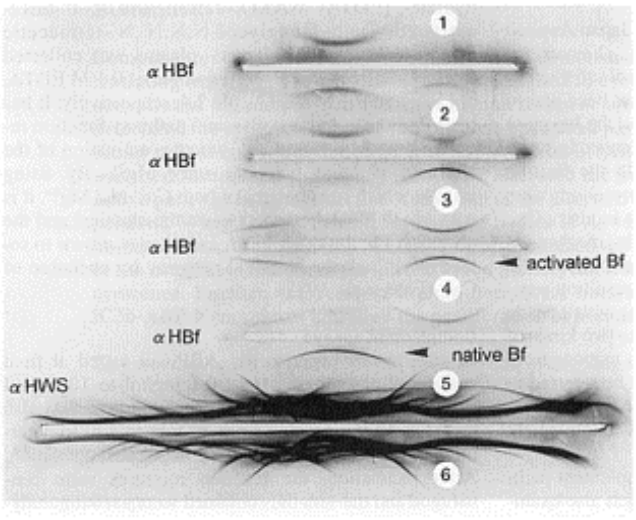

Figure 3b: Immuno-electrophoresis was performed in the agar gel. Figure show the results demonstrating activated in human HumanBf fragment. 1) Non-activated Human serum 2) that administered 5 gr of MRY/day 3) 10 gr/day, 4) activated serum 15 gr/day,, 5) and 6) were MRY–free human serum

is made to make precipitin line for 90 min and the dialyzed by physiological saline and then desolation and dried and colored by blue dye for visualization. Immuno-electrophoresis was prepared for 90 min, followed by incubating with anti-human whole serum and specific for C3 element. The specific anti human complement component antibodies were kindly presented by s by Dr Syunnosuke SAKAI, Cancer Research Institute of Kanazawa University, Japan (Figure 3a and 3b) [23,24].

Discussion and Conclusion

In animal model showed that MRY was the safe for the animal safety test. Four weeks after the administration, granulocyte and lymphocyte ratio was regulated as modulate and neutral. The emotional hormone adrenaline and dopamine level in serum also regulated neutral that dose dependent and reversible manner. The mechanism of this regulation had triggered by starting complement activation by absorbed MRY fragment through intestinal wall to the lymph [25].

We proposed an idea that MRY exhibited remedy effect for the regulation of immune competent cells via controlling complement component. Direct evidence was proved convert complement element by the technic, immune-electrophoresis.

Conflict of Interest

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

Acknowledgement

Declared none.

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