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Review

Utility and Safety of LPS-based Fermented Flour Extract as a Macrophage Activator

YOSHIE TANIGUCHI[1,2,3], NORIKO YOSHIOKA[1,2,3], TAKASHI NISHIZAWA[1,4], HIROYUKI INAGAWA[1,2,4,5], CHIE KOHCHI[1,2,3,4,6] and GEN-ICHIRO SOMA[1,2,3,4,6]

[1]*Institute for Health Science, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima-shi, Tokushima, 770-8514;*
[2]*Nonprofit Organization Linking Setouchi Innate Immune Network (LSIN), 388-1 Satohzukanishi, Kamiita-cho, Itano-gun, Tokushima, 771-1342;*
[3]*Department of Integrated and Holistic Immunology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, kida-gun, Kagawa, 761-0793;*
[4]*Macrophi Inc., 2217-44 Hayashi-cho, Takamatsu-shi, Kagawa, 761-0301;*
[5]*Department of Applied Aquabiology, National Fisheries University, Nagatahon-machi, Shimonoseki-shi, Yamaguchi, 759-6595;*
[6]*Institute for Drug Delivery System, Tokyo University of Science, Yamazaki, Noda-shi, Chiba, 278-8510, Japan*

Abstract. *The immune system is part of the homeostasis system. Our research is focused on ways to maintain health, with an emphasis on the role of macrophages. We have hypothesized that tissue macrophages form a systemic network which we believe contributes to the homeostasis system, and have named it the 'macrophage network.' This network creates a dynamic equilibrium situation where macrophages control homeostasis. Our research is based on this macrophage network theory as we believe that the innate immune system provides the foundation for the homeostasis system. As part of our research, we have demonstrated that macrophage activation can provide protection and therapeutic effects for various diseases. Therefore, we have also focused on lipopolysaccharide (LPS). We proved that the LPS of Pantoea agglomerans (which we have named IP-PA1) was useful in preventing various health disorders and in restoring health when administered via the oral or transdermal route. We also developed a 'fermented flour extract', which consists largely of IP-PA1. For LPS to become a valuable commodity, it is very important to provide assurance about safety (when administered orally or transdermally) to build confidence. For this reason, we tested fermented flour extract (in which the major component was IP-PA1) to confirm that it was safe. The results of these safety trials confirmed that oral and transdermal administration of fermented flour extract was very safe. Thus, we believe that fermented flour extract is a new substance that will have applications in health food, cosmetics, animal feeds, fisheries feeds and drugs industries.*

Correspondence to: Gen-Ichiro Soma, Institute for Health Science, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima-shi, 770-8514, Japan. Tel/Fax: +81 886028103, e-mail: sma5628@tokushima.bunri-u. ac.jp

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Significance of Macrophages in Homeostasis Systems

A homeostasis system is possessed by all organisms as a mechanism for keeping the internal environment constant. The concept of homeostasis was developed as a general fundamental of life by American biologist W.B. Cannon in 1929. Portions of the theory were first developed in 1854 by C. Bernard who stated, "Maintaining life requires that the internal environment remain constant." The nervous system and endocrine system are well known as part of the homeostasis system. As the immune system receives external stimulation and has functional responses, we believe that it is also part of the homeostasis system.

Currently, etiological studies make up the major part of medical studies. As etiological studies focus on the causes of diseases, the outcome has been the development of medical treatments and drug medicines. Currently, there is growing awareness of the importance of disease prevention, and this

will require new concepts and methods. We need new hypotheses for answering the question of which systems or technologies can maintain and improve health. A greater understanding of how to maintain health may also allow us to prevent or cure diseases such as lifestyle-related diseases, autoimmune disease and cancer.

Our research is focused on ways to maintain health, with an emphasis on the role of macrophages. Macrophages were discovered by llya llich Mechnikov in 1882, and after 120 years of study, macrophages are known to have functions of phagocytosis, chemotaxis, adherence, clearance of foreign substances and to play an important role in recognition and effector phases. Additionally, it appears that macrophages have a role in homeostasis, which goes beyond their normally understood narrow role as part of the immune system (1).

Macrophages play the initial and core part of immune system processes. They are at the forefront of reception of information from the external environment to which they adjust. The immune system can be divided into two categories: innate immunity and acquired immunity. It is thought that both systems together contribute to control the homeostasis system. Acquired immunity is only developed in vertebrates and is based on the biophylaxis of antigen-antibody reactions in which T- or B-cells (involved in the production of antibodies) play the central role. By contrast, innate immunity is an immune system possessed by all multicellular organisms; this system eliminates foreign substances or waste products independently of antibodies. Innate immunity is very important as it not only has a role in the biophylaxis system of the immune system, but also plays a role in homeostasis. Because innate immunity is possessed by all multicellular organisms, we have focused on macrophages, which are the key cells in innate immunity as they augment biophylaxis. There have been reports to indicate that animals that are deficient in macrophages have a variety of problems (2-5). These reports suggest that macrophages contribute to the regulation of homeostasis in organisms and are fundamental for maintaining health.

Macrophage Network Theory

In the past, the immune system has only been considered as a biophylaxis system preventing infections from foreign substances. However, Burnet suggested in 'Clonal Selection Theory' that ''the process was initially concerned not with defense against infection but with the maintenance of cellular integrity of the body.'' In his "Cellular Immunology'' (6), this concept has proven factual. For example, the secretion of preopiomelanocortin from the anterior lobe of the hypophysis in response to stress augments production of glucocorticoid, and brings on potent immune suppression. These stress reactions promote infection. In addition, cytokines, in particular interleukin (IL)-1 and tumor necrosis factor (TNF) are endogenic pyrogenic substances that raise set points of body temperature. In this way, there is no doubt that molecules such as cytokines, for example, are closely associated with the nervous and endocrine systems in homeostasis systems.

For the reasons mentioned above, and from the perspective that the immune system plays an essential role in homeostasis, we believe that the innate immune system is very important. It is for these reasons that we have focused on macrophages. Macrophages receive external information, become self activated and express membrane-bound type TNF (proTNF). They communicate with neighboring macrophages through proTNF. Meanwhile, macrophages send information and are controlled by proTNF. Thus this action works in two directions (7). We have hypothesized that tissue macrophages receive local environmental information and communicate with neighboring macrophages by juxtacrine, and thus form a systemic network. We believe this network contributes to homeostasis, and have named it the "macrophage network." This network creates a dynamic equilibrium where macrophages control homeostasis (8-10). Our research is based on this macrophage network theory as we believe that the innate immune system provides the foundation not only for differentiation and development but also for the homeostasis system (for example, the nervous system or endocrine system) (8-10). As part of our research, we have demonstrated that macrophage activation can provide protection and therapeutic effects for various diseases including cancer.

Macrophage network theory holds the promise of providing new answers to historically intractable problems related to health and immunity. However, first it is necessary to understand the characteristics of tissue macrophages and the signaling system between macrophages.

IP-PA1 and Fermented Flour Extract as a Macrophage Activator

To better define the homeostasis system, we have studied the molecular basis of functions in macrophages. We have also focused on lipopolysaccharide (LPS) (part of the exterior cell wall of Gram-negative bacteria) because this substance has strong adjuvanticity. In the original research on LPS, Coley reported that Gram-negative bacteria had antitumor effects (11). Based on his findings, he administered Gram-negative bacterial components to his cancer patients and accumulated data on antitumor effects (12).

In 1991, we discovered a substance in a water extract of flour which activated macrophages after being administered orally or intradermally. The active substance was LPS derived from *Pantoea agglomerans*, a Gram-negative bacterium which grows symbiotically with wheat (13). We proved that the LPS of *P. agglomerans* (which we have named IP-PA1) was useful in preventing various health disorders and restoring health

Table I. *Species of Gram-negative bacteria used in food processing.*

Scientific name of bacteria	Name of food (producing district)
Acetobacter aceti	Vinegar (worldwide)
Zymomonas mobilis	Tequila (Mexico)
Xanthomonas campestris	Xanthan gum (worldwide)
Acetobacter xylinum	Nata de coco (Philippines)
Acetobacter orientalis	Caspian Sea yogurt (Caucasus)
Enterobacter cloacae	Sarapao (Thai)
Pantoea agglomerans	Fermented rye bread (Northern Europe)

Table II. *LPS content in health food and Chinese herbs.*

	LPS** (μg/g)	Amount of LPS in daily dosage***
Health food (Scientific name)*		
Wakame seaweed (*Undaria pinnatifida*)	21.20	31.80
Wheat bran	8.80	132.00
Wheat germ	7.50	180.00
Shiitake mushroom (*Lentinus edodes*) powder	2.00	
Barley sprouts****	2.95	88.50
Barley (young leaf) powder	0.42	2.52
Chinese herbs***** (Scientific name)		
Sinomenium stem (*Sinomenium acutum*)	600	
Ginseng (*Panax ginseng*)	50	
Bupleuri radix (*Bupleurum scorzoneraefloiurm*)	40	
Licorice (*Glycyrrhiza glabra*)	30	
Pueraria root (*Pueraria lobata*)	30	

*Commercially available health food in Japan; samples were suspended in distilled water and heated for 5 hours at 60˚C. **Endospecy (LPS-specific detection) was used to determine the LPS content in the samples. ***The amount of LPS was calculated based on the daily recommended dosage. ****Food used by ulcerative patients. *****Chinese herbs that were commercially available in Japan were washed and dried; these samples were suspended in distilled water and heated for 5 hours at 60˚C.

when administered *via* the oral or transdermal route (14-20). Likewise, Braun-Fahrlander *et al*. reported that the amount of LPS exposure is inversely correlated with the frequency of asthma attacks in a wide range of epidemiological studies (21). Moreover, toll-like receptor 4 (TLR4)- or MyD88-deficiency in mice caused aggravation of ulcerative colitis (UC) provoked by dextran sodium sulfate (DSS). In addition to this, it was reported that UC provoked by DSS was inhibited in wild-type (WT) mice by orally administered LPS (22). For the afore mentioned reasons, we think that LPS plays an direct important role in the homeostasis system.

Based on these hypotheses, our research has helped clarify certain aspects of the homeostasis system. Utilization of LPS contributes to the control of the macrophage network and helps maintain homeostasis. Because of our concern about how to administer LPS in a safe manner, we developed a 'fermented flour extract', which consists largely of IP-PA1. Fermented flour extract was made from wheat flour fermented by *P. agglomerans* with biotechnology and can provide IP-PA1 safely and inexpensively. Presently, the utilization of fermented flour extract has progressed to the stage where it is being injected into chicken feeds, farmed fish feeds, human health foods and cosmetics.

Is LPS only an Endotoxin (Toxic Material) or Is it a Substance of Value?

Under certain medical situations LPS behaves as an 'endotoxin.' This occurs when the LPS is administered intravenously, rather than orally or through the skin. In the blood stream, LPS induces the production of cytokines, even at doses as low as 4 ng/kg, and causes excessive inflammation; in humans this can result in endotoxin shock, hypotension or sepsis (23). Therefore, drugs and medical products administrated intravenously are required to be free of LPS. Until recently, the major focus of study on LPS has been the deleterious effects and toxicity that is ascribable to strong cytokine induction.

The toxicity of LPS that is caused by strong systemic cytokine induction occurs only when it is in the blood stream. Other immunostimulators also induce production of cytokines and express bioactivity on a scale that is equivalent to that of LPS. An example is lactic acid bacilli. Lactic acid bacilli, which are Gram-positive bacteria, contain peptide glycan in their cell walls. It has been reported that intravenous administration of lactic acid bacilli also causes shock in the same way as LPS (24). However, these negative aspects are not emphasized for lactic acid bacilli. It does not make sense that LPS is considered as a toxic substance while lactic acid bacilli are not. Lactic acid bacilli have been used for a number of purposes and have been administered orally and transdermally, and it is well known that these routes are very safe. Accordingly, we believe oral and transdermal administration of LPS is safe. There are many healthy foods that that employ Gram-negative bacteria during processing; these contain Gram-negative bacteria and LPS (Tables I and II). This demonstrates empirically that oral intake of LPS and Gram-negative bacteria can be safe.

As described above, in contemporary society, improvements in hygiene correspond to a reduction in the consumption of LPS from the environment and it has been reported that this phenomenon is associated with an increase in allergic diseases (25, 26). In other words, historically, the inadvertent consumption of LPS and Gram-negative bacteria has kept our immune system in balance and helped with homeostasis. Consequently, we believe that the intake of LPS and Gram-negative bacteria would have a beneficial effect on protection from allergic diseases in contemporary society. It is a novel concept that in societies where there has been

Table III. *Safety trials for fermented flour extract – Somacy-FL100 and Somacy-FP100.*

Study	Contents	Amount	Results	Testing lab
Reverse mutation test	Using 5 strains to detect genetic mutations	156.3-5,000 μg/plate	No aberration	Ina Research, Inc. (Japan)
Chromosome aberration test	Using Chinese hamster cultured cells (CHL/IU)	625-5,000 μg/ml	No aberration	Ina Research, Inc. (Japan)
Single-dose toxicity study	Rats [Crl:CD(SD)], administered once.	2,000 mg/kg	No toxicity	Ina Research, Inc. (Japan)
28-Day repeated-dose toxicity study	Rats [Crl:CD(SD)], administered for 28 days.	10-1,000 mg/kg	No toxicity	Nihon Bioresearch Center, Inc. (Japan)

We entrusted these safety trials to testing laboratories.

an excessive level of hygiene, LPS may be a valuable substance for keeping the immune system balanced and for maintaining homeostasis. This is the reason we developed a fermented flour extract which contains LPS.

Safety of Fermented Flour Extract

As mentioned above, LPS has historically been part of human diets. Nevertheless, most research has only emphasized the toxicity of LPS. For LPS to become a valuable commodity, it is very important to provide assurance about its safety, to build confidence. For this reason, we tested fermented flour extract to confirm that it was safe (Tables III and IV).

Fermented flour extract has been developed for chicken farming and farmed fish (Somacy-SL100), for human health foods (Somacy-FL100, Somacy-FP100), and for cosmetics (Somacy-CL010). We performed safety trials of fermented flour extract which was to be used in human health foods and for cosmetics (Tables III and IV).

First, we performed safety trials on the fermented flour extracts, Somacy-FL100 and Somacy-FP100. The initial safety trials included a reverse mutation test using 5 strains to detect genetic mutations, a chromosome aberration test using Chinese hamster cultured cells (CHL/IU), a single-dose toxicity study using rats [Crl:CD(SD)], and a 28-day repeated dose toxicity study using rats [Crl:CD(SD)]. The practical maximum dose from fermented flour extract (Somacy-FL100 and Somacy-FP100) is slightly less than 1 mg/kg (weight)/day. In the single-dose toxicity study, the test dose was 60,000 times higher than the practical dose from the extract, and the 28-day repeated dose toxicity study was carried out at a dose 30,000 times higher. Toxicity was not observed in either test.

Next, we performed safety trials of the fermented flour extract, Somacy-CL010. These safety trials included a reverse mutation test using 5 strains to detect genetic mutation, a chromosome aberration test using Chinese hamster cultured cells (CHL/IU), a single-dose toxicity study using rats [Crl:CD(SD)], a skin irritation study (temporality and superfetation) using rabbits (Kbl:JW), a conjunctival irritation study using rabbits (Kbl:JW) and a skin sensitization study (Adjuvant and Patch Test methods), a skin phototoxic study (Morikawa methods), and a skin photosensitization study (Adjuvant and Strip Methods) using Hartley guinea pigs. The practical concentration of fermented flour extract Somacy-CL010 is almost 1 mg/1 g end-product. These safety tests used extra large doses and only one adverse reaction was observed, even when the concentration was 75 times higher than the practical concentration. The sole adverse reaction was actinic erythema observed in the skin irritation study (superfetation).

The results of these safety trials confirmed that oral and transdermal administration of fermented flour extract was very safe.

Prospects for Fermented Flour Extract

We clarified the bioactivity of IP-PA1 and fermented flour extract in previous research. IP-PA1 and fermented flour extract had the same effect of lowering blood low density lipoprotein (LDL) levels in WHHL rabbits as hyperlipemia (27). In addition, it had the same protective effect on gastric ulcer provoked by indomethacin or a stress-induction operation in mice (28), and a suppressive effect of type I diabetes sideration in NOD mice (29). Thus, IP-PA1 and fermented flour extract provide protective and therapeutic effects for a variety of diseases. Furthermore, these substances have a suppressive effect on pain caused by acetic acid in mice and have a therapeutic effect in cancer (MethA, MH134) in mice (30). These substances also provide therapeutic effects for human cancer (31-33). Additionally, as mentioned above, it is clear that oral and transdermal administration of fermented flour extract is very safe. Thus, we believe that fermented flour extract is a new substance that will have applications in health food, cosmetics, animal feeds, fisheries feeds and drugs industries (9).

Our discovery of IP-PA1 and the fermented flour extract was

Table IV. *Safety trial for fermented flour extract Somacy-CL010.*

Study	Contents	Amount	Results	Testing lab
Reverse mutation test	Using 5 strains to detect genetic mutations	1.22-5,000 μg/plate	No aberration	BOZO Research Center, Inc. (Japan)
Chromosome aberration test	Using Chinese hamster cultured cells (CHL/IU)	5,000 μg/ml	No aberration	BOZO Research Center, Inc. (Japan)
Single dose toxicity study	Rats [Crl:CD(SD)], dose administered once	2,000 mg/kg	No toxicity	Nihon Bioresearch Center, Inc. (Japan)
Skin irritation study (temporality)	Rabbits (Kbl:JW), dose administered for 24 hours	Bulk powder used at 2.5-50μg/ml	No irritation	Nihon Bioresearch Center, Inc. (Japan)
Skin irritation study (superfetation)	Rabbits (Kbl:JW), dose administered for 23 hours, test carried on for 14 days	Bulk powder used at 2.5-250 μg/ml	Bulk powder: No aberrations. Actinic erythema at maximum dose.	Nihon Bioresearch Center, Inc. (Japan)
Conjunctival irritation study	Rabbits (Kbl:JW), dose administered once in eye	100 μl/eye	No irritation	Nihon Bioresearch Center, Inc. (Japan)
Skin sensitization study (Adjuvant and Patch Test methods)	Using Hartley guinea pigs	Concentrate solution (1 g/ml)	No sensitization	BOZO Research Center, Inc. (Japan)
Skin phototoxic study (Morikawa methods)	Using Hartley guinea pigs	Concentrate solution (1 g/ml)	No phototoxicity	BOZO Research Center, Inc. (Japan)
Skin photosensitization study (Adjuvant and Strip Methods)	Using Hartley guinea pigs	Concentrate solution (1 g/ml)	No photosensitization	BOZO Research Center, Inc. (Japan)

We entrusted these safety trials to testing laboratories.

a result of research on the application of the macrophage network theory. We think there can be a wide range of beneficial effects of IP-PA1 and it can be used as a tool to analyze macrophage functions in homeostasis systems. When macrophage functions are clarified, IP-PA1 may provide more effective protective and therapeutic methods for various diseases.

Finally, attention is being paid to therapies based on innate immunity. Among them, it is reported that a TLR agonist has the ability to provide protection and/or therapy for cancer, allergic diseases and viral infections (34). LPS has been shown to be a TLR4 agonist (34). Therefore, LPS has future potential for providing protective and therapeutic effects for various diseases. Ultimately, LPS may become better known for its advantageous properties than for its role as a toxic substance.

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